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Abstract:

Significant progress has been made on all aspects of the originally described work and the project is proceeding as planned and is ahead of schedule. The mesencephalic progenitor cells have been successfully cloned, characterized and shown to share numerous phenotypic characteristics with primary mesencephalic cells. We have also shown that the clones can be cryopreserved. In addition, the cloned and converted cells respond to traditional DA neurotoxins possess Nurr1 like normal primary DA neurons. We have developed assays for calbindin and microglia that will be applied to the clones. In addition, we have successfully grafted the cloned cells in the striatum of rats with 6-hydroxydopamine lesions and demonstrated proof of principle...i.e., progenitor cells converted to DA neurons by hematpoietic cytokines can attenuate behavioral asymmetries and survive as DA neurons in a fashion similar to that seen when primary mesencephalon is used. This later experiment was the primary focus of the original proposal. Finally, as a result of the knowledge gained through our work with cytokines in DA neuron development, we have expanded our studies into the role cytokines play in DA neuron degeneration with the intention of demonstrating that pro-inflammatory cytokines are a risk factor for Parkinson's disease.

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FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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S M Carney Sept. 25, 2001

PI - Signature Date

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Year 2 Annual Report:

5: Introduction:

We have shown that the ventral mesencephalon (VM) of rats contains progenitor cells (PC)/stem cells that can be converted into dopamine (DA) neurons in culture following incubation with the hematopoietic cytokines, interleukin-1 (IL-1), leukemia inhibitory factor (LIF), and glial cell line derived neurotrophic factor (GDNF). We plan to clonally expand these cells and characterize them with the intent of eventually identifying the signals which regulate the phenotype for DA neurons. This will be accomplished by testing the following hypothesis:

Cytokines drive the conversion of rat VM PCs into functional DA neurons that when grafted, attenuate the rotational asymmetries of the 6-hydroxy-DA lesioned rat.

6: Body of Report: Progress in Year 02:

Significant progress has been made on the project in its second year. This progress, which is organized around the original statements of work, may be summarized as follows:

Statement of Work: (Copied from original proposal)
Year 1-2.5;

The mesencephalic progenitor cells (PC) will be cloned using limiting dilutions to identify the cell line that will respond to interleukin-1 with the highest percentage of dopamine (DA) neurons.

The conversion media which currently contains the unknown substances fetal calf serum, striatal conditioned media, and mesencephalic membrane fragments, will be systematically evaluated to determine if these unknowns can be replaced by Neural Basal Media with the N-2 spplement, known trophic molecules including glial cell-line derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and known extracellular matrix molecules (ECMs)/cell adhesion molecules (CAMs), respectively.

Full characterization of converted culture phenotypes including all DA neuron markers and other cell types anticipated in these cultures (gabaergic cells, neurofilament cells, astrocytes, microglia, and oligodendroglia).

The effects of the sequential addition of the cytokines IL-1, LIF, and GDNF will be assessed on DA neuron conversion and morphological development.

Seeding density studies will be performed to determine the optimal density for plating which could provide a critical threshold for differentiation.

Perfecting the rotary culture technique as well as the paraffin embedding techniques needed to characterize aggregated and differentiated progenitor cells using this technique.

1- The progenitor/stem cells have been successfully cloned yielding two cell lines called MPC-8 and MPC-9. As we reported in our preliminary findings last year, these two cell lines, and

in particular MPC-9, were isolated using the limiting dilution technique and characterized for their conversion to dopamine (DA) neurons in response to interleukin-1 (IL-1). Both MPC-8 and 9 were shown, via immunoreactivity for tyrosine hydroxylase (TH), to have approximately 98% TH immunoreactive (ir) cells. Of the 24 cell lines isolated from the clonal expansion, many contained THir cells and some (MPC-4) contained few THir cells (MPC-4 was predominantly immunoreactive for the astrocyte marker GFAP).

2- MPC-9 has been used extensively in the lab this year (this clone has also been sent to 4 other laboratories in Marburg Germany, U. of Miami, NIDA, Baltimore, and to Tim Collier's lab at Rush. All groups have found the clone stable). MPC-9 has been shown to be stable in our hands. Exposure to the full cytokine cocktail which includes IL-1, leukemia inhibitory factor (LIF), and glial cell line-derived neurotrophic factor (GNDF) reveals stable conversion to THir cells at ~ 75%. The potential reasons for the discrepancy between the initial conversion rate of 98% and 75% after numerous passages are discussed in the appended manuscript. We feel the primary reasons are cell clumping and transient expression of TH in non-dopaminergic cells.

3- MPC-9 in response to the cytokine mixture has been shown to be immunoreactive to, DA, dopa-decarboxylase, TH, the DA transporter (DAT), and to make DA released into the culture mixture (assessed using mass spec [240 pg/ml]). We have also characterized these cells for GFAP, nestin, and Gal-C (oligodendorcyte marker). We have just completed developing calbindin immunocytochemistry and will be assessing the cultures for this co-localized protein this fall. We have also characterized the cultures for Nurr-1 which is also present in DA neurons (see appended manuscript). We are currently working up OX-42 (microglia stain) and once the protocol is complete, will apply it to the clones (this fall). We have stained the cultures for MAP-2, a general neuronal marker, and the cultures are immunoreactive. We have not started to run the GAD stain yet for GABAergic neurons.

4- We originally required the presence of striatal conditioned media and mesencephalic membrane fragments to induce conversion. We have now been able to replace this very complicated support media with 98% Neural basal media (NBM) containing 2% N-2 supplement. Growth is not as pronounced, but more than adequate. This is preferred over the "black-box" approach used in the past. We will be testing extracellular membrane fragments in the next year, however, as we feel these proteins are very helpful in completing the morphological development of the neurons. The cells are now very well developed, but we feel we can get greater process extension with ECMs and nCAMs.

5- We have not done extensive seeding density studies and may not pursue this. One of the problems we have encountered is that the clones are very adherent and tend to form neurospheres very readily. Thus, they adhere readily regardless of density, and changing seeding density would not change this tendency to form neurospheres. This has presented problems, however. Because of the adherent nature of these cells and there tendency to form spheres, it is difficult to separate them without significant trypsin digestion which leads to

poor viability. We have thus been forced to use mechanical trituration which is "hard" on the cells (yields lower viability). We will be trying alternative methods in the this upcoming year which will include low levels of trypsin followed by trituration with progressively narrower bore pipettes to reduce stress to the cells.

6- We had initially abandoned the need for rotary culture because we discovered that 5 hour exposure to the cytokines in a test tube was adequate for conversion (see enclosed manuscript). As a result, we were not going to pursue this procedure. However, recent studies with Caryl Sortwell in Tim Collier's lab (Co-investigator on this project) has shown promising results with primary mesencephalic cultures and caspase inhibitors. Because of the low survival of the clones in animals, (see below) which we think is the result of apoptosis and the trituration problems alluded to above, we now plan to pursue a rotary culture procedure. In the presence of the caspase inhibitor, we feel we can grow the clones to small spheres and then load those spheres into a Hamilton syringe for delivery to a graft site. We feel this will address many of the problems we have encountered in grafting these cells (see appended manuscript for discussion of viability and low survival).

7- The enclosed published manuscript demonstrates significant progress on the sequential delivery of the cytokines (these results are currently being collected). Our initial results suggest that IL-1 is the key signal. Exposure to LIF or IL-11 or GDNF helps mature the cells once exposed to IL-1, but none of these molecules induce conversion to THir cells in the absence of IL-1. We have also determined that IL-1 incubation of the cells leads to the development of gp-130 imunoreactivity. Gp-130 is the receptor for LIF and IL-11. Thus, Il-1 turns on gp-130 allowing response to LIF or IL-11. We plan to perform the same set of studies with the ret receptor for and GDNFR for GDNF. As a result of these initial studies, we feel we no longer need to include IL-11 in the cytokine mixture. We now feel that the enhanced conversion we saw in the initial non-cloned cells when both IL-11 and LIF were present was due do to increased stimulation of the gp-130 receptor produced by having both ligands in the media. We have chosen to now use only LIF.

SUMMARY: We feel we have made excellent progress on all aspects of the study included in the statement of work to be completed by the mid-point of year 2. The work is proceeding well and is ahead of schedule.

Related to Statement of Work Year 2.5-3.5 (Copied from original proposal)
Year 2.5-3.5;

Once identified, the converted cultures will be characterized for their functional similarities to natural mesencephalic cultures containing DA neurons. These tests will include:

1- their ability to take DA into the cell out of the media.

2- their ability to release DA into the media.

3- their ability to respond to known trophic molecules including GDNF, BDNF, platelet derived neurotrophic factor (PDGF), insulin-like growth factor (ILGF), and basic fibroblast growth factor (βFGF) as well as dibutyryl cAMP.

4- their ability to respond in vitro to known DA neurotoxins including 6-hydroxydopamine,

1-methyl-4-phenyl- 2,3,5,6-tetrahydropyridine (MPTP), and tumor necrosis factor (TNF).

1- We are just beginning to start this work. Aside from our efforts to show the similarities between MPC-9 and primary mesencephalic cultured neurons described above (similar cell markers) we plan to perform a full characterization of other aspects of their phenotype (e.g., Nurr-1 and Calbindin). We have preliminary results on the response of these cells to known DA neurotoxins (enclosed color confocal image). As seen, cytokine exposed MPC-9 cells have well developed cell bodies and processes. Addition of known DA neurotoxins including levodopa (50 μM), MPP+ (10 μM) and tumor necrosis factor alpha (TNFα, 50 ng/ml) leads to overt cell loss. We have not had the opportunity to initiate cell counts in these cultures, but it does appear that they are susceptible to these neurotoxins.

SUMMARY: We have begun this work and it appears that indeed the cloned cells respond to known DA neurotoxins in a fashion analogous to primary mesencephalic cultures. Within the next 18 months we will complete this characterization as outlined in the statement of work.

Statement of Work for Year 3.5-end of 4 (copied from original proposal);

The progenitor cells will be transfected with the BAG retrovirus marker for permanent identification,
The progenitor cells will be grafted into the striata of young and old animals with unilateral 6-hydroxydopamine lesions and allowed to survive for various lengths of time (2,4, and 6 months) during which the following behavioral assessments will be performed:
1- apomorphine-induced ipsilateral rotation
2- amphetamine-induced contralateral rotation
3- skilled paw-reach test.
The animals will be sacrificed and the grafts assessed for:
1- PC survival
2- DA neuron differentiation
3- the presence of other neuron and glial types in the cultures
The behavioral data will be statistically tested to identify if correlations exist between the behavioral and cellular outcome measures.
The data will be summarized, written up and published.

1- We have made significant progress on this specific aim. As discussed in last year's report, we had preliminary data of the effectiveness of this procedure when using the non-cloned cells and we were just beginning to pursue the use of the clones. We went ahead and implemented this aim using the MPC-9 clone and the results from this study are described in detail in the appended manuscript. As was true of the preliminary study, the cloned cells did indeed attenuate rotation in lesioned rats. Thus:
   a. The grafted cells were successfully grafted.
   b. Grafted cells exposed to cytokines for 5 hours in a test tube and then grafted, developed into DA neurons and the phenotypes (THir) was stable for two months.
   c. Clones that were not exposed to cytokines prior to grafting did not convert to the

THir phenotype.

d. Converted clones attenuated amphetamine-induced rotational behavior to the same extent as did primary mesencephalic cells, albeit at a slower rate. Regardless, the eventual degree of rotational attenuation was of the same magnitude as primary mesencephalic cells.

e. The viability of the clones in the graft site was lower than that of the primary cells.

f. The cell loss during the grafting procedure was significantly greater for the clones than the primary cells (trituration issue discussed above).

2- We have not yet characterized the cells in vivo for other cell markers. We will likely repeat this study using rotary cell culture neurospheres incubated with caspase inhibitors as describe above. At that time we plan to perform a full characterization.

SUMMARY: We have made excellent progress on this aim and feel that we have achieved proof of principle. In our mind this proof of principle was significant and we submitted the manuscript to Science. It, however, was rejected and an expanded version of the manuscript is now in review at J. Neuroscience (see enclosed).

OTHER WORK STIMULATED BY THE ORIGINAL CONCEPT:

We have been intrigued by the role cytokines play in DA neuron development and feel that the whole field of cytokines and their involvement in DA neuron development and phenotype regulation is a potentially rich field. Work complementary to the current work has been initiated. We have included the Specific Aims page from the 2 year NIEHS proposal which starts Oct. 1 , 2000 and the abstract from the DOD proposal which we just submitted for a concept exploration proposal (CEP) for the September, 20, 2000 deadline for the next round of the Neurotoxin Exposure/treatment program. Hopefully, the reader will recognize that the following two studies together with the present work on cytokine-induced conversion of progenitor cells, will provide invaluable information of the overall role of cytokines in the CNS. The other two projects may be described as follows:

FUNDED NIEHS Study

A. Specific Aims:

The mechanism(s) which initiates dopamine (DA) neuron degeneration in Parkinson's disease (PD) is unknown, but a cycle of neural degeneration from oxidant stress induced by free radical formation that is self-perpetuating has been hypothesized. Recent evidence from our laboratory suggests that the pro-inflammatory cytokines interleukin-1 beta [IL-1β] and tumor necrosis factor alpha [TNFα], but not interferon gamma IFN-γ, can initiate DA neuron degeneration. In preliminary studies, lipopolysaccharide (LPS), a well known inducer of pro-inflammatory cytokines and a mediator of the toxic effects of Gram(-) bacteria, dose-dependently increased TNF-α, IL-1, and nitric oxide (NO) and selectively killed DA neurons in primary mesencephalic cultures. This cell loss was associated with a reduction in the anti-apoptotic protein Bcl-2. In addition, we found that maternal injection of LPS into a gravid rat at embryonic (E) day 10 (the time just prior to DA neuron birth), led to a dramatic reduction in DA neurons in the embryonic mesencephalon at E15. This diminution

in DA neurons was still present at post-natal (P) day 10 and was associated with altered DA biochemistry. Further analysis revealed that TNF-α and IL-1β were up-regulated in the brains of the pups at P10 suggesting that inflammatory processes triggered *in utero* were still ongoing at P10. These data suggest that environmental exposure to Gram(-) bacteria during gestation may lead to underdevelopment of the nigro-striatal DA system leaving the affected organism with fewer DA neurons at the start of life. This heretofore unstudied potential environmental mechanism for the etiology of PD will be examined via the following two specific aims of this Exploratory/Developmental Proposal.

<u>Specific Aim 1:</u> **Systemic injection of LPS (10k units/kg) into a gravid female rat at E10 produces decreased DA neuron function and DA cell number in her offspring.** Brains will be harvested from embryos or offspring sacrificed at E 10.5, 11, 12, 12.5, 13, 13.5, 14, 15, 18, and P1, 10, 20, and 40. DA and its primary metabolite homovanillic acid (HVA), will be assessed in the striata using HPLC and used as an index of DA function. DA neuron counts in the SNpc of the animals will be quantified using stereology. TNF-α, IL-1β, and IFN-γ protein expression will be assessed using Enzyme-Linked Immuno-Sorbent Assays (ELISA) and used as an index of pro-inflammatory cytokine activation and its change over time. Striatal glial cell line-derived neurotrophic factor (GDNF) will be assessed using ELISA to determine if LPS reduces DA neuron development by interfering with the activity of this well known DA neurodevelopmental factor. The mother's rectal temperature, white blood cell (WBC) counts, and serum cytokine levels will also be monitored and correlated with the offspring's DA function, DA cell counts, and striatal GDNF so that the temporal relationship between systemic infection and fetal response can be evaluated. It is hypothesized that relative to control animals, LPS will induce compromised DA function in the offspring that will be associated with increased pro-inflammatory cytokines and decreased GDNF.

<u>Specific Aim 2:</u> **Exposure of primary mesencephalic cultures to LPS will increase pro-inflammatory cytokines which will kill DA neurons via apoptosis.** Indices of DA neuron survival including tyrosine hydroxylase (TH), DA, and the DA transporter (DAT) will be assessed immunocytochemically in primary mesencephalic cultures following exposure to LPS. Primary mesencephalic culture TNF-α, IL-1β, and IFN-γ (ELISA assessments), and NO (fluorometric analysis) as well as Bcl-2, and other members of the Bcl-2 protein family (Bcl-X_L and Bax), will be assessed (using Western blot) and correlated with the indices of DA neuron survival and apoptosis-mediated DA cell loss (confocal microscopy of TH and propidium iodide). It is hypothesized that increased apoptosis in DA neurons subsequent to LPS exposure will be correlated with increased TNF-α and IL-1β, decreased Bcl proteins, and increased Bax and NO. This hypothesis will be tested further by demonstrating prevention of DA cell loss using IL-1β and TNFα antagonists as well as inhibitors to NO synthase.

Implementation of the specific aims of this Exploratory/Developmental Proposal will establish if exposure to Gram(-) infections while *in utero* represents a possible mechanism for the development of PD in later life. Support for this hypothesis would serve as the basis for longer term studies on the effects of LPS including the effects of repeated exposure in adulthood, increased sensitivity to pro-

inflammatory cytokines, and increased sensitivity to oxidant stress. The successful completion of these studies would encourage epidemiologic studies on the relationship between life-time Gram(-) illness and the risk of PD.

COMMENT: It is important to recognize that this work was a direct consequence of our work with cytokines and progenitor cells. We started to study the effects of LPS in gravid female rats because we felt that increasing the amount of IL-1 in the brain of a fetus just prior to the birth of DA neurons would increase the number of DA neurons. The LPS study was logical based on the notion that IL-1 is a critical trigger to progenitor cell conversion to DA neurons. In actuality, we found the exact opposite.

Title and abstract from the DOD CEP award just submitted:

TNFα/IL-1β-induced Dopamine Cell Loss: A Potential Model for the Pathogenesis of Parkinson's Disease

Evidence from our laboratory suggests that brain injection of the pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) produces an apparent loss of DA neurons. Numerous lines of evidence from other laboratories also suggest that these cytokines are involved in the pathogenesis of Parkinson's disease (PD). We propose to evaluate the injection of TNFα and/or IL-1β into rat brain as a new animal model of PD. Traditional dose-response and time-course studies will be performed following the injection of TNFα and/or IL-1β into the medial forebrain bundle or the lateral ventricle of the brain. Analysis of DA and its metabolites, indices of the DA phenotype (tyrosine hydroxylase and calbindin), glial measures (astrocyte and microglia markers), and alterations in TNFα and IL-1β levels following cytokine injection will confirm or refute our operational hypothesis; **infusion of TNFα and/or IL-1β into the brains of rats produces a partial DA neuron loss in the substantia nigra similar to that seen in PD patients.** The probable involvement of pro-inflammatory cytokines in the CNS effects of germ and chemical warfare, brain trauma, and other CNS disorders further extends the potential usefulness of the collected data once generalized to other systems.

COMMENT: This submitted proposal, we feel, now completes the initial characterization of the role of pro-inflammatory cytokines within the CNS. Based on our preliminary findings (see below), it appears that pro-inflammatory cytokines may play a role in adult DA neuron loss as well since injection into brains of IL-1 and TNF induces DA neuron loss in a calbindin-sparing fashion. Successful completion of this work along with the progenitor cell proposal and the NIEHS funded work will help us to complete a global model we are proposing.

Global Model for Cytokines in DA neuron phenotype regulation:
It is clear that pro-inflammatory cytokines play a part in DA neuron development as well as death. We are now starting to believe that pro-inflammatory cytokines play a dual role. Thus, low levels of inflamation lead to low levels of IL-1 production, the conversion of progenitor cells to DA neurons,

and their migration into the inflamed site (the damaged mesencephalon). The role of pro-inflammatory cytokines under these conditions is to initiate repair of the substantia nigra. However, if the insult to the substantia nigra is severe causing a high level of pro-inflammatory cytokines to be produces, the result is greater cell death. The pro-inflammatory cytokines may thus be part of an overall regulatory scheme designed to maintain the substantia nigra on one hand, but also be detrimental to DA neurons if the insult is too great. This model is in its initial stages of development and the work over the next couple of years will determine if there is any validity to it.

Figure Legends:

FIGURE 1: Confocal images of DA neurons using TH as a marker (red) and calbindin (green) in primary cultures exposed to TNFα. A, B, and C are cultures exposed to Hank's balanced salt solution (HBSS) while D-F are exposed to 100 ng TNFα. Light yellow in F is a THir cell that is also expressing calbindin. This figure demonstrates that we have now perfected calbinding immunocytochemistry and that it can be used to characterize the cloned progenitor cells. Figure also depicts the notion that TNFα is toxic to DA neurons.

FIGURE 2: Depicts MPC-9 clone stained for TH following exposure to the cytokine cocktail (cytokine only), cytokines followed by 50 μM levodopa, 10 μM MPP+, and 100 ng TNFα. Note that all three know DA neurotoxins, reduce the number of DA neurons in a fashion typical of the effect these toxins have on normal primary mesencephalic cultures. These cultures are currently being assessed for cell counts to complete this component of work.

FIGURE 3: Effects of 100 ng TNFα alone and in combination with 100 pg IL-1β. The animals were injected with these cytokines into the medial forebrain bundle and after seven days sacrificed and perfused. The sections were stained for TH and then photgraphed under low power (left side) and higher power (right side). As can be seen, TNFα and TNFα with IL-1β produce a loss of THir cells in the mesencephalon.

FIGURE 4: Essentially the same experiment as in Figure 3 except that these are ipsilateral and contralateral sides of the same animals injected with TNFα into the MFB. It demonstrates our ability to perform calbindin immunocytochemistry (green cells) in vivo as planned for the current proposal (red is TH). Note that TNFα reduces THir cells and increases calbindin-ir cells and that the loss of THir cells is predominantly in cells not up-regulating their calbindin.

7: Key Research Accomplishments:

- Have successfully cloned mesencephalic progenitor cells.
- Cloned progenitor cells have numerous characteristics in common with primary mesencephalic cells including DA production, DA release, TH, DDC, DAT, Nurr-1, and response to known DA neurotoxins.
- Have simplified conversion media to known chemicals.
- Have reduced the need for the fourth cytokine IL-11 previously used in the conversion.
- Have shown that simple incubation of progenitor cells in cytokines in a test tube induces a

stable conversion of the cells to DA neurons.

- Have shown that the cloned cells can be successfully cryopreserved.
- Have successfully grafted cloned progenitor cells into a rat with 6-OHDA lesion and attenuated amphetamine-induced rotational behavior in a fashion similar to that seen after grafting primary mesencephalic cells.
- Have developed immunocytochemical procedures for staining calbindin and OX-42 for microglia.

8: Reportable Outcomes:

Manuscripts/abstracts Published, in Review, or in Preparation:

Directly related to the work and appended:

Potter, E.D., Z.D. Ling and P.M. Carvey: Cytokine-induced conversion of mesencephalic-derived progenitor cells into dopamine neurons. Cell Tiss. Res. 296:235-246, 1999.

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Carvey, Paul M., ZaoDung Ling, Carol E. Sortwell, Mark R. Pitzer, Susan O. McGuire, A. Storch, and Timothy J. Collier: Mesencephalic progenitor cells converted to dopamine neurons by hematopoietic cytokines: A source of cells for transplantation in Parkinson's disease., J. Neuroscience, Submitted.

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Vu, TQ, Ling, ZD, Ma, SY, Robie, HC, Tong, CW, Chen, EY, Lipton, JW, and Carvey, PM. Pramipexole attenuates the dopaminergic cell loss induced by intraventricular 6-hydroxydopamine. Journal of Neural Transmission, 107:(2), 159-176, 2000.

Goetz, CG, Vu, TQ, Carvey, PM, and Leurgans, S. Posthypoxic myoclonus in the rat: Natural history, stability, and serotonergic influences. Movement Disorders, 15(1): 39-46. 2000.

9: Conclusions:

Progress made on this project is significant and is actually ahead of schedule. With few exceptions, the work that was described has been completed or is in progress. The few components of the study that have not been addressed or have been dropped (e.g. rotary cultures) have been shown to be inappropriate based on scientific thinking about the project as it has progressed. In addition, the support for this work has led our laboratory to explore the role cytokines play in the DA system in other areas including risk factors for Parkinson's disease. Thus, in addition to the work directly related to the funded proposal from the DOD, the concepts originated in this grant have led to a significant expansion of ideas as it relates to PD. Moreover, developing an understanding of cytokines in the DA system has far reaching implications for military personnel in the field. Although our work has focused on the DA system, the appreciation for the role cytokines play in CNS function in general can be extrapolated to other neurodegenerative diseases as well as the CNS consequences of germ warfare and chemical warfare where we are convinced that pro-inflammatory cytokines are also involved.

10: References:

None aside from those included in the submitted manuscripts.

11: Appendix:

Manuscripts and abstracts included:

1- Potter, E.D., Z.D. Ling and P.M. Carvey: Cytokine-induced conversion of mesencephalic-derived progenitor cells into dopamine neurons. Cell Tiss. Res. 296:235-246, 1999.

2- Carvey, Paul M., ZaoDung Ling, Carol E. Sortwell, Mark R. Pitzer, Susan O. McGuire, A. Storch, and Timothy J. Collier: Mesencephalic progenitor cells converted to dopamine neurons by hematopoietic cytokines: A source of cells for transplantation in Parkinson's disease., J. Neuroscience, Submitted.

3- Carvey, P.M., Z.D. Ling, : Interleukin-1 Induces Nurr-1 Expression and the DA Phenotype in Mesencephalic Progenitor Cells. Soc. for Neuroscience Abstracts, 133.1. October 1999.

4- Carvey P.M., Z.D. Ling, C.E. Sortwell, M.R. Pitzer, and T.J. Collier. Progenitor cells converted to DA neurons by cytokines can be grafted and cryopreserved. American Society for Neurotransplantation and Repair Abstracts 7:32. 2000.

5- Also included are glossy photos with the figure legends described above.

Cell Tissue Res (1999) 296:235–246

REGULAR ARTICLE

Elizabeth D. Potter · Zao Dung Ling · Paul M. Carvey

Cytokine-induced conversion of mesencephalic-derived progenitor cells into dopamine neurons

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Abstract We have previously shown that a combination of the cytokines interleukin (IL)-1, IL-11, leukemia inhibitory factor (LIF), and glial cell line-derived neurotrophic factor (GDNF) can convert rat fetal (E14.5) mesencephalic progenitor cells into tyrosine hydroxylase (TH)-immunoreactive (ir) neurons in vitro. The experiments described here characterize the mesencephalic progenitor cells and their cytokine-induced conversion into dopamine (DA) neurons. For all experiments, we used bromodeoxyuridine (BrdU)-ir cultures of (E14.5) mesencephalic progenitor cells that had been expanded at least 21 days. We first demonstrated that IL-1 induced DA neuron conversion in mesencephalic progenitors, but not in striatal progenitors ($P<0.001$). Thus, these cells should be classified as lineage-restricted progenitors, and not omnipotent stem cells. To further characterize cell populations in these cultures, we used monoclonal antibodies against Hu (an early marker for neurons), growth-associated protein (GAP)-43 (a marker for neuronal process extension), TH (a marker for DA neurons), and glial fibrillary acidic protein (GFAP, a marker for astrocytes). We assessed (E14.5) mesencephalic progenitor cell cultures (plated at 125,000 cells/cm^2) incubated in the cytokine mixture (described above) or in complete media (CM, negative control). Following 7 days incubation, GFAP-positive cells formed a nearly confluent carpet in both types of cultures. However, numbers of Hu-ir and GAP-43-ir cells in the cytokine-incubated cultures far exceeded those in CM-incubated controls ($P=0.0003$, $P=0.0001$, respectively), while numbers of TH-ir cells were 58-fold greater in the cytokine-incubated cultures versus CM-incubated controls. The TH phenotype persisted for 7 days following withdrawal of the differentiation media. Numerous double-labeled cells that were BrdU-ir and also TH-ir, or Hu-ir and also TH-ir, were observed in the cytokine-incubated cultures. These data suggest that cytokines "drive" the conversion of progenitor cells into DA neurons.

Key words Transplantation · Parkinson's disease · CNS fetal development · CNS differentiation · Neurotrophic factors · Rat

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Introduction

Progenitor cells are pluripotent, self-renewing cells that are lineage restricted (Anderson 1989; McKay 1997; Ray et al. 1997; Stemple and Mahanthappa 1997). Thus, unlike omnipotent stem cells, which theoretically can differentiate into any cell type, progenitor cells can differentiate into only a restricted set of cell types (McKay 1997; Ray et al. 1997). Numerous studies have shown that self-renewing cells residing in the subependymal zone of the adult and fetal brain can migrate and then differentiate into neurons and glia (Cattaneo and McKay 1990; De Vitry et al. 1980; Fredricksen et al. 1988; Levison and Goldman 1993; Lois and Alvarez-Buylla 1993, 1994; Luskin 1993; Luskin and McDermott 1994; Morshead and van der Kooy 1992; Winkler et al. 1998). Hynes and colleagues (1995a,b) have shown that mesencephalic floor plate cells induce the differentiation of mesencephalic subependymal cells into a specialized phenotype, the dopamine (DA) neuron. These studies also showed that if mesencephalic subependymal cells were transplanted to a more rostral location, none of these subependymal cells differentiated into DA neurons (Hynes et al. 1995a,b), suggesting that local environmental factors play a role in the differentiation of mesencephalic subependymal cells into DA neurons. These data also support a hypothesis of lineage restriction, with respect to differentiation into a specialized cell type (the DA neuron) that

is specific to location along the rostral-caudal axis of the neural tube (Hynes et al. 1995b). Yet clearly this local environmental effect is constrained by the innate genetic predisposition of the mesencephalic subependymal cells to differentiate into neurons and glia (and not other cell types). Thus, these data demonstrate that local environmental factors and a genetic predisposition limiting differentiation to specific cell types together play a role in the differentiation of a subpopulation of the mesencephalic subependymal cells into DA neurons (Hynes et al. 1995a,b; Jessel and Dodd 1990; Kilpatrick and Bartlett 1993; McKay 1997; Richards et al. 1992; Temple 1989).

Recently, many researchers have cultured fetal and adult subependymal cells isolated from the striatum and mesencephalon using media supplemented with the mitogen epidermal growth factor (EGF) (Reynolds et al. 1992; Reynolds and Wiess 1992; Ptak et al. 1995; Svendsen et al. 1995). Subependymal cells cultivated in the EGF-supplemented media are self-renewing and capable of division for several months (Reynolds and Weiss 1996; Ptak et al. 1995; Svendsen et al. 1995). When grown in poly-l-lysine (PLL)-coated tissue culture wells in serum-containing media, the mesencephalic and striatal subependymal cells differentiate into neurons and glia. The cultured striatal subependymal cells differentiate into the glia and GABAergic neurons normally found in the striatum (Reynolds et al. 1992). However, cultured mesencephalic subependymal cells rarely differentiate into DA neurons (Svendsen and Rosser 1995). This finding suggests that additional environmental factors play a critical role in DA neuron differentiation. In support of this, we have recently shown that E14.5 rat mesencephalic subependymal cells can be induced to express the DA neuron phenotype by exposure to certain cytokines (Ling et al. 1998). When we incubated fetal mesencephalic subependymal cells in media containing interleukin-1 (IL-1), IL-11, leukemia inhibitory factor (LIF), glial cell-line derived neurotrophic factor (GDNF), striatal cell-conditioned media (15%) and flash-frozen mesencephalic cellular fragments (10%), the subependymal cells differentiated into cells that: (1) morphologically resembled mature DA neurons; (2) were immunoreactive to the DA neuron markers tyrosine hydroxylase (TH), dopa-decarboxylase (DDC), DA transporter (DAT), and DA neurotransmitter; but were (3) immunonegative to the noradrenergic (NE) neuron marker, DA-beta-hydroxylase (DβH). The converted TH-ir cells represented approximately one-half of the neurofilament-immunoreactive (NF-ir) cells. In addition, numerous glial fibillary associated protein (GFAP), and GAL-C-immunoreactive cells (markers for astrocytes and oligodendrocytes, respectively) were found in the cytokine-exposed cultures. These data suggest that hematopoietic cytokines, which regulate the differentiation and expansion of cells in the hematopoietic system, can perform a similar function in the central nervous system (CNS).

Although our recent experiments demonstrate that cytokines are capable of inducing DA neuron differentiation, many questions concerning the differentiation process remain. First, can cytokines induce the DA neuron phenotype in subependymal cells isolated from other CNS locations, or is the cytokine-induced DA neuron conversion unique to mesencephalic subependymal cells? If subependymal cells from many CNS locations can be converted into DA neurons following exposure to the same cytokines, this argues for classifying these subependymal cells as stem cells (Reynolds and Weiss 1996). If, on the other hand, only mesencephalic subependymal cells can be converted through cytokine exposure into DA neurons, then lineage restriction has already occurred, and these subependymal cells should be classified as progenitors. Second, do cytokines induce the conversion of the subependymal cells into neurons, or do the cytokines convert neurons into the DA neuron phenotype? Third, once cytokine exposure converts mesencephalic subependymal cells into DA neurons, will the converted cells revert to a non-DA phenotype following cytokine withdrawal? If the DA phenotype remains stable following cytokine withdrawal, this could argue in favor of cytokine involvement in the differentiation process, as these particular cytokines may be absent in the adult brain. The following set of experiments was designed to answer these three questions.

Materials and methods

Reagents used

Glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) were gifts from Amgen (Thousand Oaks, CA), and the monoclonal Hu antibody was a generous gift from Dr. Henry M. Furneaux, Sloan Kettering Institution (New York, NY). Epidermal growth factor (EGF) was purchased from Sigma Chemical (St. Louis, MO), and the remaining cytokines were purchased from R & D Systems (Minneapolis, MN). Dulbecco's modified Eagle's media (DMEM), F-12 nutrient mixture (F-12), and Hanks' buffered saline solution (HBSS), fetal calf serum (FCS) and B27 supplement were purchased from Gibco (Grand Island, NY). Normal horse serum (NHS), normal goat serum (NGS), penicillin-streptomycin (Pen-Strep), trypsin, deoxyribonuclease (DNase), transferrin, putrescine, sodium selenite, progesterone, insulin, glucose, glutamine, Triton X-100, 3-3' diaminobenzidine (DAB), nickel sulfate, imidazole (IA), sodium acetate, Trizma base, sodium and potassium phosphates were all purchased from Sigma Chemical (St. Louis, MO). The anti-rat monoclonal tyrosine hydroxylase (TH) primary antibody (1:5000) was purchased from Incstar (Stillwater, MN). The anti-rabbit polyclonal TH primary antibody (1:250) was purchased from Chemicon (Temecula, CA). The anti-mouse monoclonal growth-associated protein (GAP)-43 antibody (1:2000) was purchased from Boehringer Mannheim (Indianapolis, IN). The anti-mouse monoclonal glial fibrillary associated protein (GFAP) antibody (1:2000) was purchased from Sternberger Monoclonal (Baltimore, MD). The biotinylated anti-mouse secondary antibody (IgG), fluorescein (FITC)-avidin, Texas red-streptavidin, and avidin-biotin complex (ABC) were purchased from Vector Labs (Burlingame, CA). Neuropore used to prepare cultures for BrdU labeling was purchased from Trevigen (Gaithersburg, MD). BrdU labeling was performed using a kit (No. RPN20) from Amersham (Arlington Heights, IL).

Subependymal cell isolation, expansion, and preparation

The fetal mesencephalic subependymal cells (E14.5) were isolated according to methods previously described (Ptak et al. 1995; Svendsen et al. 1995). Striatal progenitor cells were isolated according to the methods of Reynolds and Weiss (1992). Mesencephalic and striatal cultures were expanded according to the methods described by

Svendsen et al. (1995). Briefly, the mesencephalic or striatal tissues were micro-dissected from fetal rats under sterile conditions, incubated in 0.1% trypsin for 30 min at 23°C, incubated in DNase (40 μg/ml) for 10 min at 37°C, gently triturated into a cell suspension using a fire-polished pipette, and plated into 25-cm^2 flasks (400,000 cells/ml) in 5 ml "expansion media" [F12/DMEM 1:3; 100 U penicillin/100 μg streptomycin (Pen-Strep)/ml; 2 ml B27 per 100 ml media; 20 ng/ml EGF] for 21 days. The yield from this procedure was approximately 50–100 "proliferation spheres" per cm^2. These cells have been passaged up to 4 months by feeding the cells once a week with expansion media, and gently triturating the spheres and reseeding the cells into new 25-cm^2 or 75-cm^2 flasks (filled with 10 ml expansion media) every 2 weeks.

Cytokine incubation of subependymal cells

Following incubation in expansion media, the proliferative spheres were triturated and the cells added (125,000 trypan blue-excluding cells/cm^2) to PLL-coated 48-well plates and incubated 7 days in either complete media, "cytokine-enriched" media, or "differentiation media." Complete media (CM) consisted of DMEM/F12 1:1, 10% FCS, and 1% Pen-Strep. Cytokine-enriched media consisted of CM and each of the cytokines listed in Table 1. The concentrations of cytokines used were 5 times the ED_{50} values recommended by the manufacturers (see Table 1). The differentiation media consisted of CM supplemented with the cytokines IL-1 (200 pg/ml), IL-11 (1 ng/ml), LIF (1 ng/ml), and GDNF (1 ng/ml), freeze-fractured mesencephalic membrane fragments (10%w/v) and striatal conditioned media (15%). We have previously demonstrated (Ling et al. 1998) that while IL-1 induces the DA neuron phenotype in mesencephalic subependymal cultures, a combination of IL-1, IL-11, LIF, and GDNF, mesencephalic fragments, and striatal conditioned media increased numbers of converted DA neurons, and also produced a mature morphology in the converted DA neurons. The mesencephalic membrane fragments were prepared as previously described (Ling et al. 1998) by plating out E14.5 mesencephalic primary cultures at 250,000 cells/cm^2. After 3 days, the complete media were removed and cultures were washed in CM, then placed in a –80°C freezer for 1 h and thawed at 23°C three successive times. The membrane fragments were collected and stored at –20°C until use. The striatal-conditioned media were collected after 72 h from E14.5 primary cultures of the lateral ganglionic eminence (striatal primordia) plated at 250,000 cells/cm^2 and grown in defined media [DM: DMEM/F12 1:1, 1% Pen-Strep., transferrin (100 μg/ml), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), insulin (25 μg/ml), glucose (0.63 g/100 ml), and glutamine (2nM)].

Immunocytochemistry

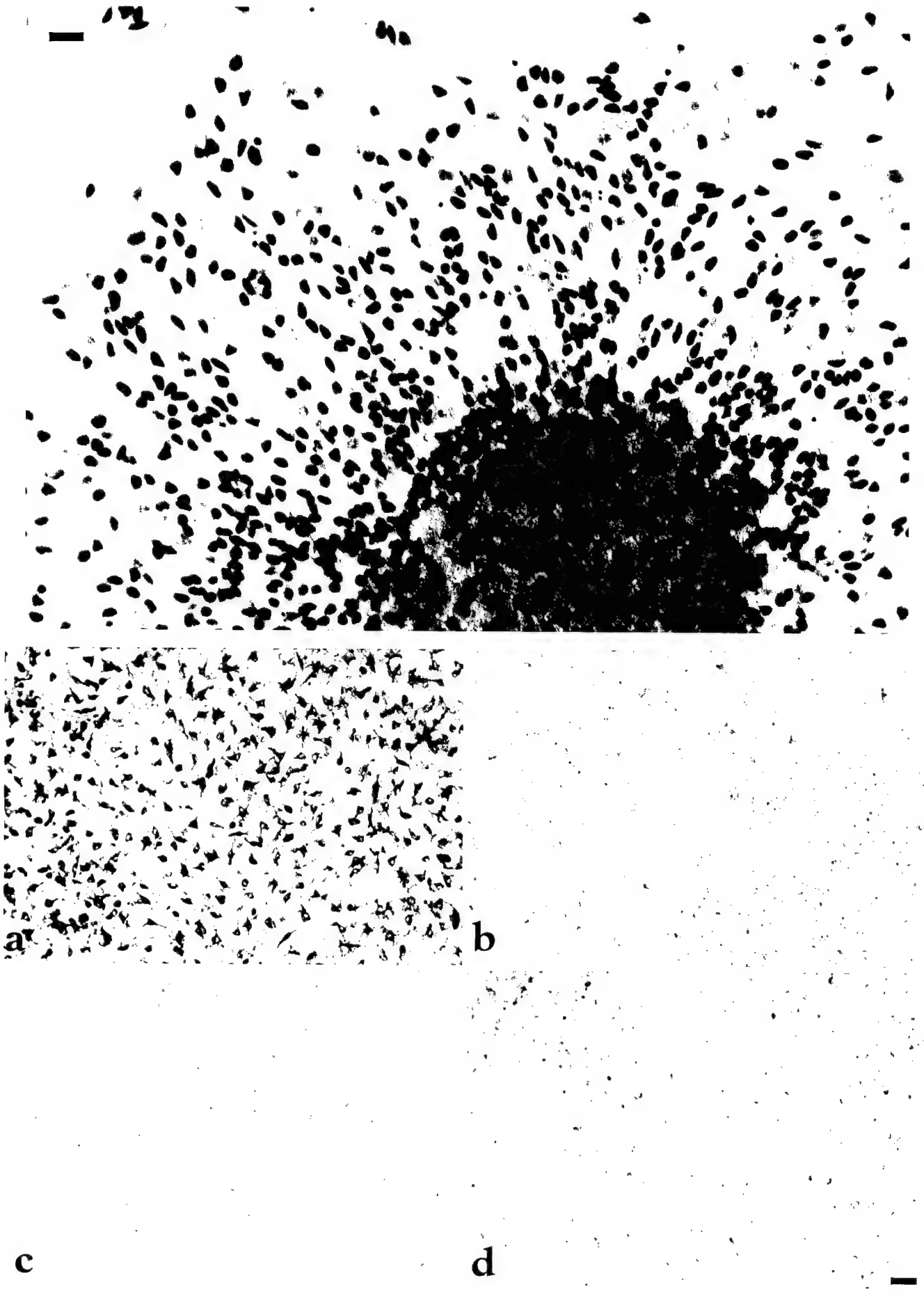
Following the prescribed incubation the cultures were fixed for 20 min and TH immunocytochemistry was performed according to methods described earlier (Ptak et al. 1995). Briefly, the cultures were fixed in 3.7% formalin/phosphate-buffered saline (PBS) solution for 20 min at 23°C, incubated 1 h in blocking solution consisting of Triton X-100 (0.2%)/normal horse serum (NHS:10%)/TRIS-buffered saline (TBS), and incubated in monoclonal anti-rat TH primary antibody (1:5000; Incstar) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG (0.5%), and incubated 1 h in avidin-biotin complex (ABC). The TH stain was developed using DAB (0.05%) and nickel sulfate (2.5%) during a 1-min incubation at room temperature. For Hu (1:4000) immunocytochemistry, the methods were the same. Hu is an RNA-binding protein that acts as a specific marker for neurons and for progenitor cells that are committed to express the neuronal phenotype (Barami et al. 1995; Marusich et al. 1994). For GFAP (1:2000) immunocytchemistry, the methods were the same, except 5% NHS was used in the blocker. GFAP is a marker for astrocytes. For (GAP)-43 (1:2000) immunocytochemistry, the methods were the same, except 5% NHS and no Triton was used in the blocker. GAP-43 is a marker for neurons extending processes. BrdU labeling was performed using Vector and Amersham reagents and a modified protocol from Amersham (Arlington Heights, IL) kit (RPN-20). BrdU labeling is a marker for cell proliferation. Double labeling with BrdU and polyclonal anti-rabbit TH antibody was used to assess whether progenitor cells have converted into TH neurons. Double labeling with Hu and polyclonal anti-rabbit TH antibody was used to assess whether TH-positive cells were also Hu-positive neurons.

For BrdU and TH double labeling, the cultures were fixed as described above. To prepare the formalin-fixed cultures for BrdU labeling, the cells were incubated 5 min in 70% EtOH, 10 min in PBS, and 10 min in 0.2% Triton/TBS. The cells were then incubated in a 50:50 solution of the monoclonal BrdU antibody (1:100) and Neuropore (Trevigen) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG:0.5%, and incubated 1 h in Texas red streptavidin (1:100). Following three washes in PBS, the cultures were incubated 1 h in a blocking solution consisting of Triton X-100 (0.2%)/NGS(5%)/TBS, and incubated in the polyclonal anti-rabbit TH primary antibody (1:250; Chemicon) overnight at 4°C. The cultures were incubated 1 h in anti-rabbit IgG:0.5%, and incubated 1 h in FITC-avidin (1:300).

For Hu and TH double labeling, the cultures were fixed as described above, incubated 1 h in blocking solution consisting of Triton X-100 (0.2%)/NHS(10%)/TBS, and incubated in monoclonal anti-rat Hu primary antibody (1:4000) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG(0.5%), and incubated 1 h in Texas red streptavidin (1:100). Then following three washes in PBS, the cultures were incubated 1 h in a blocking solution consisting of Triton X-100 (0.2%)/NGS(5%)/TBS, and incubated in polyclonal anti-rabbit

Table 1 Effect of cytokines in conversion of progenitors into DA neurons [*"M"* mesencephalic progenitors (125,000 cells/cm^2) incubated 7 days in complete media supplemented with the designated cytokine (see below), *"S"* striatal progenitors (125,000 cells/cm^2) incubated 7 days in complete media supplemented with the designated cytokine, *IL-1* interleukin-1 (200 pg/ml), *IL-2* (2 ng/ml), *IL-3* (1 ng/ml), *IL-4* (5 ng/ml), *IL-6* (200 pg/ml), *IL-9* (100 pg/ml), *IL-11* (1 ng/ml), *CSF* colony-stimulating factor (1 ng/ml), *TGFβ* transforming growth factor beta (1 ng/ml), *TGFα* (1 ng/ml), *LIF* leukemia inhibitory factor (1 ng/ml), *EGF* epidermal growth factor (100 ng/ml), *SCF* stem cell factor (40 ng/ml), *βFGF* beta fibroblast growth factor (100 ng/ml), *EPO* erythropoietin (0.5 U), *INFγ* interferon gamma (100 ng/ml), *TNFα* tumor necrosis factor alpha (200 pg/ml), *GDNF* glial-cell-line-derived neurotrophic factor (1 ng/ml), *BDNF* brain-derived neurotrophic factor (1 ng/ml), *Zero* nothing added, +++ cells positive for the dopamine neuron marker TH are present, – no TH immunoreactivity]

Cytokine progenitors	"M"	"S"
IL-1	+++	–
IL-2	–	–
IL-3	–	–
IL-4	–	–
IL-6	–	–
IL-9	–	–
IL-11	–	–
CSF	–	–
TGFβ	–	–
TGFα	–	–
LIF	–	–
EGF	–	–
SCF	–	–
βFGF	–	–
EPO	–	–
INF-γ	–	–
TNFα	–	–
GDNF	–	–
BDNF	–	–
Zero	–	–


a
b
c
d

◀ **Fig. 1** Bromodeoxyuridine (*BrdU*) antibody staining of mesencephalic progenitor sphere plated onto poly-L-lysine (*PLL*)-coated wells and incubated 24 h in complete media. ×320. *Bar* 15 μm

TH primary antibody (1:250; Chemicon) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG:0.5%, and incubated 1 h in FITC-avidin (1:300). Following three washes in NaCl (0.15 M)/bicarbonate (0.1 M), the fluorescent stains were visualized using an Olympus Fluoview Confocal microscope.

Experiment 1

Mesencephalic subependymal cells cultivated at least 3 weeks in expansion media, were labeled 24 h with BrdU, then plated 24 h on poly-L-lysine (PLL)-coated 48-well Costar plates, fixed and incubated with the monoclonal BrdU antibody, and BrdU immunocytochemistry was performed. This experiment was performed to demonstrate the presence, or absence, of proliferating cells in the mesencephalic subependymal cell cultures used in the experiments described below.

Experiment 2

Striatal and mesencephalic subependymal cultures that had been incubated in expansion media for at least 21 days were plated onto PLL-coated 48-well Costar plates, and cultured under identical conditions in the "cytokine-enriched" media. Nineteen different cytokines were evaluated (see Table 1). Following 7 days incubation in the cytokine-enriched media, the cultures were fixed and the conversion of subependymal cells was assessed by counting numbers of TH-ir cells. This experiment was performed to determine if cytokines could induce DA neuron differentiation in striatal subependymal cell cultures, as well as in mesencephalic subependymal cell cultures.

Experiment 3

Hu, GAP-43, TH, and GFAP immunoreactivity of mesencephalic subependymal cultures was assessed 7 days after plating EGF-stimulated proliferative spheres into PLL-coated 48-well Costar plates. Hu immunocytochemistry was used to assess numbers of subependymal cells capable of differentiation that were committed to the neuronal phenotype. GAP-43 immunocytochemistry was used to assess numbers of neurons extending processes. TH immunocytochemistry was used to assess numbers of subependymal cells converted into DA neurons. The cultures were incubated in either differentiation media (see above) or CM (control) for 7 days and then fixed. Four computer-generated random fields of each well were identified on a reticle grid, and the number of Hu-immunoreactive (Hu-ir) cells was assessed and compared across treatment groups. In sister cultures on the same 48-well Costar plates, GAP-43, TH, and GFAP immunoctyochemistry was performed and the immunoreactive cell counts were similarly gathered. These assessments were performed to determine the relative cell counts of each of the four labeled cell types.

Experiment 4

To demonstrate the conversion of progenitors into TH neurons, cultures of mesencephalic subependymal cells were double-labeled using BrdU and TH antibodies. To demonstrate that TH-positive cells were neurons, these same cultures were double-labeled using Hu and TH antibodies. Proliferating mesencephalic subependymal cells were plated on PLL-coated wells and incubated in either differentiation media (see above) or CM (control) for 7 days, then fixed and double labeled using BrdU and TH antibodies, or fixed and double labeled with Hu and TH antibodies as described above. An Olympus Fluoview Confocal microscope was used to evaluate double-labeling with the fluorescent-tagged antibodies.

◀ **Fig. 2** TH immunoreactivity of a mesencephalic progenitor sphere plated onto PLL-coated wells (125 000 cells/cm^2) and incubated 7 days in CM supplemented with (**a**) and without (**c**) IL-1 (200 pg/ml). Striatal progenitors plated using the same procedure and incubated with (**b**) and without (**d**) IL-1. ×250. *Bar* 20 μm

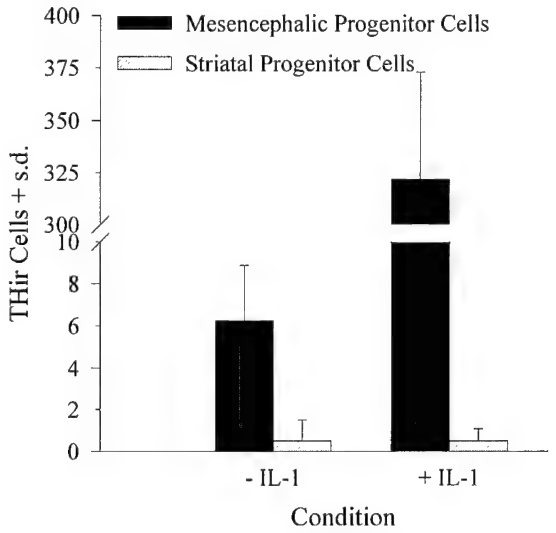


Fig. 3 Comparison of the TH-ir cell counts from the conditions depicted in Fig. 2. Mesencephalic and striatal progenitor cells were exposed to IL-1 (+IL-1; 200 pg/ml) in complete media or to complete media alone (–IL-1)

Experiment 5

Proliferating mesencephalic subependymal cells that had been incubated in expansion media for at least 21 days were plated on PLL-coated wells and incubated in the differentiation media (see above) for 7 days, after which cultures were incubated another 7 days in CM without cytokines. A set of sister cultures was incubated for 14 days in CM only. Following fixation numbers of TH-ir cells were assessed to determine whether continuous cytokine exposure was needed to maintain the DA neuron phenotype.

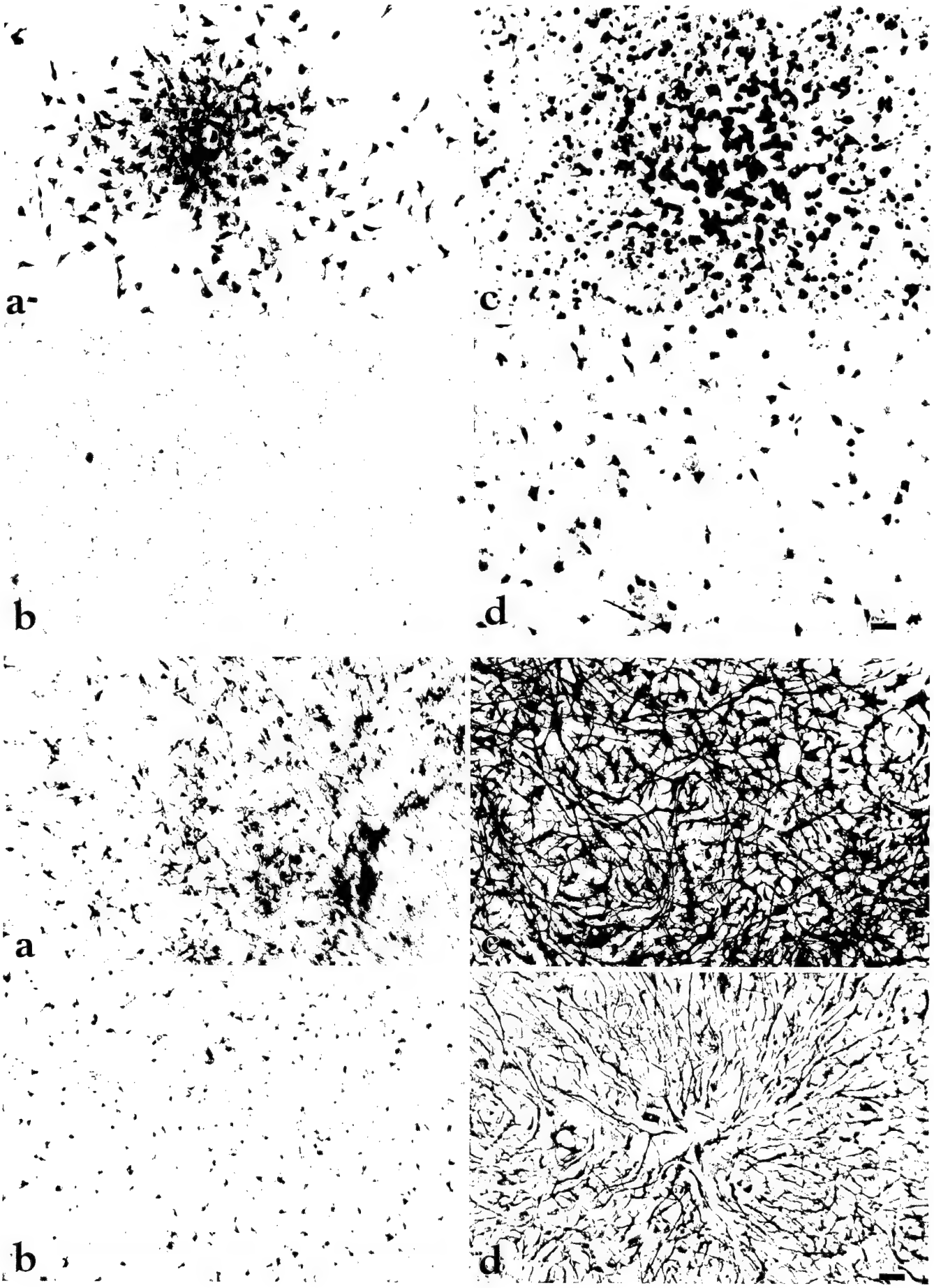
Results

BrdU labeling and immunocytochemistry of proliferating subependymal cells: experiment 1

Numerous cells in the proliferating sphere of subependymal cells were BrdU-positive, as indicated with the black DAB-nickel stain (Fig. 1). All of the spheres plated contained numerous BrdU-positive cells although not all of the cells in each sphere were labeled.

Cytokine-induced DA neuron conversion in mesencephalic and striatal subependymal cells: experiment 2

The ability of 19 different cytokines to convert E14.5 mesencephalic and E14.5 striatal fetal subependymal cells to cells immunoreactive for TH is shown in Table 1. While IL-1 (200 pg/ml) converted mesencephalic subependymal cells


a
b
c
d
a
c
b
d

◀ **Fig. 4** TH immunoreactivity of mesencephalic progenitor spheres exposed to differentiation media (**a**) or complete media (**b**) for 7 days. Hu immunoreactivity in sister cultures exposed to differentiation media or complete media for 7 days is shown in **c** and **d**, respectively. ×250. *Bar* 20 μm

into primitive TH-ir cells (Figs. 2a, 3; $F_{1,15}$=164.36; P<0.001), none of the striatal cultures incubated in the cytokines had more than two TH-ir cells (Figs. 2b, 3). In the absence of cytokines, no more than two TH-ir figures were observed in the mesencephalic and striatal progenitor cultures (Fig. 2c,d, respectively; Fig. 3).

Hu, GAP-43, TH and GFAP immunoreactivity in subependymal cells: experiment 3

In mesencephalic cultures incubated 7 days in either the differentiation media (described above), or CM without cytokines, numbers of TH-ir, Hu-ir, and GAP43-ir cells were counted. Mesencephalic progenitor cell cultures incubated 7 days in the differentiation media exhibited a dramatic maturation in cells having a neuronal phenotype (Fig. 4a), compared with cultures incubated in CM without cytokine supplementation (Fig. 4b). TH-ir cells exposed to the cytokines were far more numerous and exhibited a cell body more typical of DA neurons from primary cultures. Moreover, most of the cells contained processes. Although numerous Hu-ir cells were present in cultures incubated in CM only (Fig. 4d), the number of Hu-ir cells increased dramatically in the presence of cytokines (Fig. 4c). A similar trend was observed when GAP43-ir cell counts were assessed (Fig. 5a,b). Thus, the number of GAP43-ir cells was dramatically increased and their morphology was more variegated compared with cultures incubated with CM only. The cell count data from these cultures reflected the impression of the photomicrographs (Fig. 6). Thus, the number of TH-ir cells was increased 58-fold in the cytokine-incubated cultures relative to CM-incubated controls ($F_{1,8}$=76.77, P>0.001), while the Hu-ir and GAP43-ir cell counts were increased 6-fold and 13-fold, respectively (Fig. 6; $F_{1,8}$=55.81, P=0.0003; $F_{1,8}$=95.29, P=0.0001, respectively). TH-ir cell counts represented on average 45% of the Hu-ir and GAP43-ir cell counts. Unfortunately, the GFAP-ir cells formed a confluent carpet of astrocytes in all cultures and were too numerous to count although it did appear that the differentiation media did affect their morphology (cf. Fig. 5c with Fig. 5d).

◀ **Fig. 5** Sister cultures of the cells seen in Fig. 4 are shown in **a–d**. GAP43 immunoreactivity in mesencephalic progenitors incubated 7 days in differentiation media (**a**) or in complete media (**b**). GFAP immunoreactivity antibody staining in differentiation media or in complete media is shown in **c** and **d**, respectively. ×250. *Bar* 20 μm

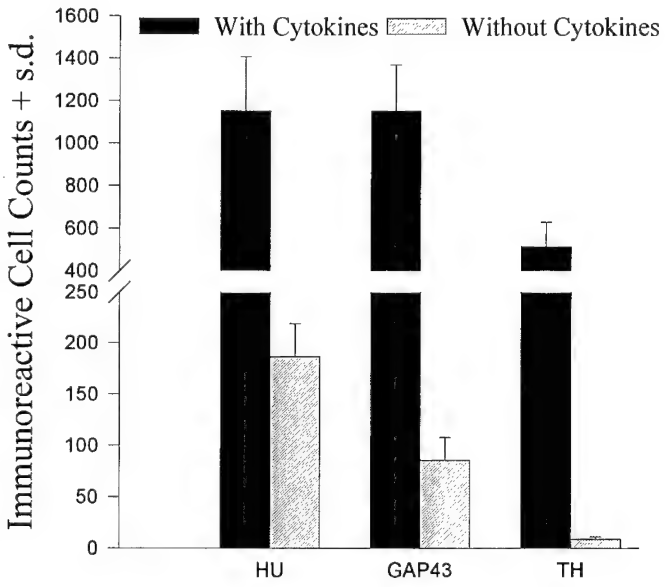


Fig. 6 Comparison of the numerical results depicted in Figs. 4 and 5 showing HU, GAP43, and TH cell counts in differentiation media (with cytokines) and CM (without cytokines)

Double-labeling with BrdU and TH immunocytochemistry, Hu and TH immunocytochemistry: experiment 4

Double labeling of progenitors with BrdU and TH fluorescent immunocytchemistry (Fig. 7a–c) demonstrated the conversion of mesencephalic progenitors into TH neurons. Most of the TH-ir cells were also BrdU-ir while many BrdU-ir cells were not TH-ir. The failure to detect BrdU immunoreactivity in all TH-ir cells likely reflects the incomplete incorporation of the BrdU label into all cells during the incubation period (note the incomplete labeling observed in Fig. 1). In contrast, double labeling was not seen in control cultures incubated in CM, as TH labeling was not present in these cultures (data not shown). Double labeling with Hu and TH fluorescent immunocytochemistry (Fig. 7d–f) demonstrated that all TH-ir cells were Hu-ir neurons. The number of Hu-ir cells that were not double labeled with TH appeared to reflect about one-half of the total Hu labeled cell population. This is in agreement with the results from the sister culture study reported in Fig. 6. Hu and TH double labeling was rarely seen in the CM-incubated controls (data not shown), as TH-ir cell numbers were extremely low in these control cultures (see Fig. 6).

Stability of cytokine-induced DA neuron conversion: experiment 5

Cells incubated in differentiation media for 7 days, followed by incubation in CM without cytokines for 7 days, maintained the expression of TH-ir cells. The cells appeared to maintain a neuronal morphology similar to that seen in cultures incubated in differentiation media for 7

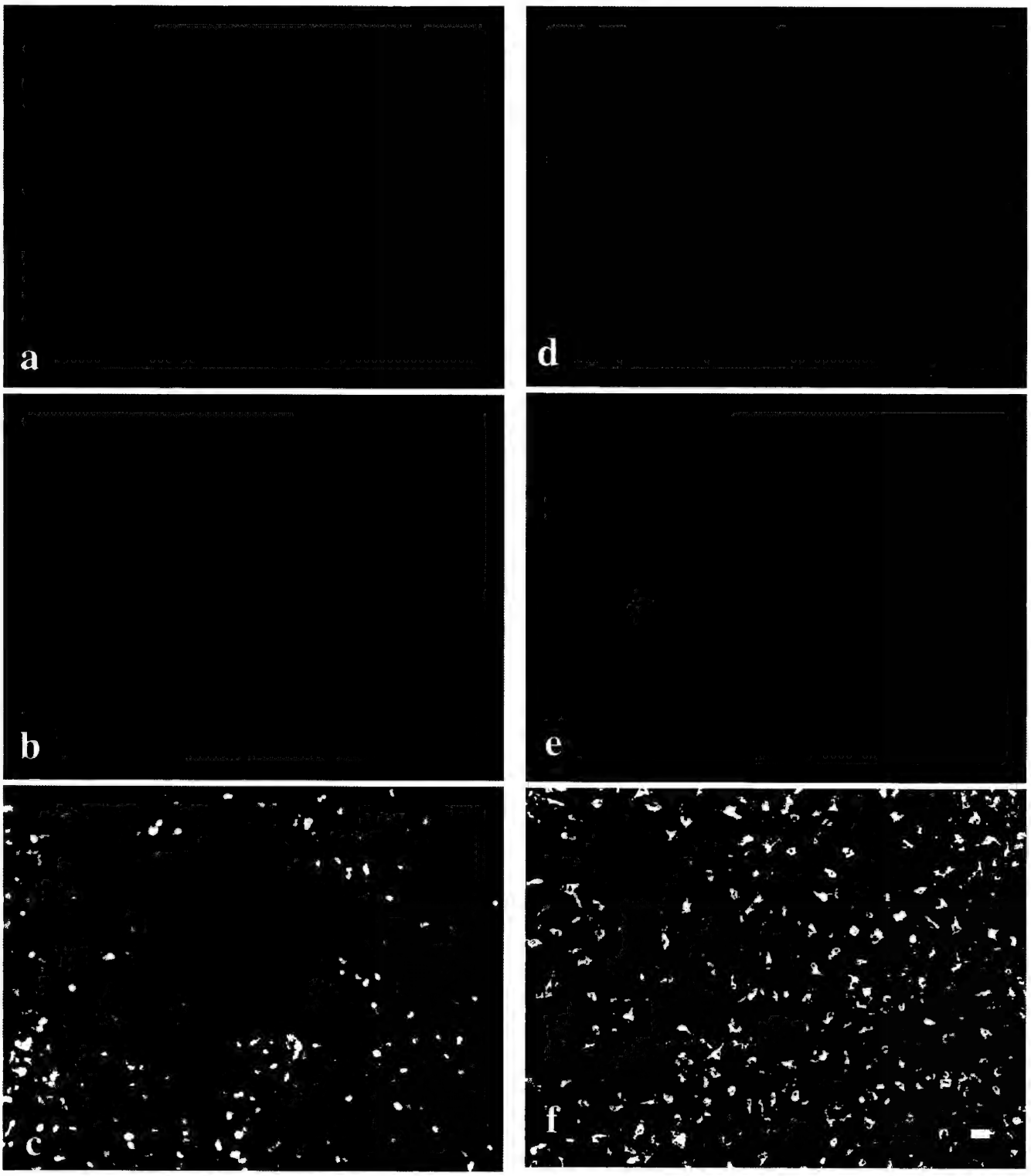


Fig. 7 Confocal images of BrdU and TH antibody staining of mesencephalic progenitor spheres plated onto PLL-coated wells (125,000 cells/cm^2) and incubated 7 days in differentiation media supplemented with cytokines [BrdU-labeled cells are red (**a**), TH-labeled cells are green (**b**), and BrdU- and TH-double-labeled cells are yellow (**c**)]. Confocal images of Hu and TH antibody staining in sister cultures of mesencephalic progenitor spheres are also shown (**d–f**) [Hu-labeled cells are red (**d**); TH-labeled cells are green (**e**); Hu- and TH-double-labeled cells are yellow (**f**)]. ×200. *Bar* 15 μm

days (cf. Fig. 8 with Fig. 4a). However, the 14-day-old TH-ir cells appeared to be slightly larger and did not have more extensive processes than cells exposed to differentiation media for only 7 days. Numbers of TH-ir cells incubated 7 days in differentiation media followed by 7 days in CM without cytokines far exceeded numbers of TH-ir cells incubated 14 days in CM without cytokines (Fig. 9;

Fig. 8 TH immunoreactivity in a 14-day-old culture of mesencephalic progenitor cells incubated 7 days in differentiation media followed by 7 days incubation in CM. ×250. *Bar* 20 μm

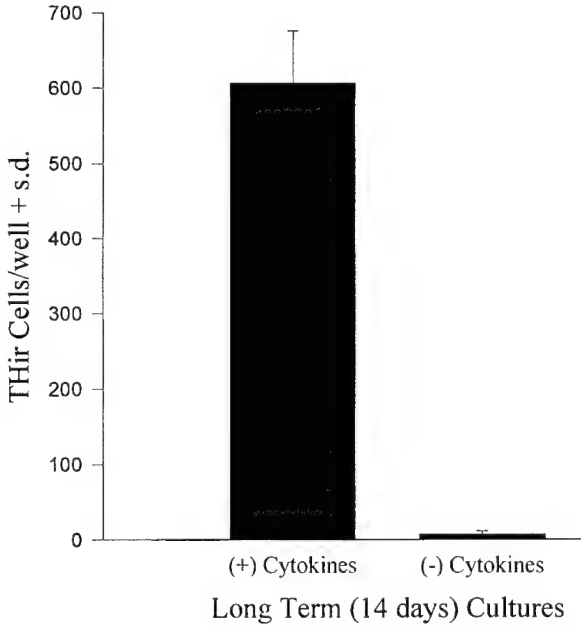


Fig. 9 Numerical results of TH-ir cell counts from cultures depicted in Fig. 8 incubated in differentiation media for 7 days followed by 7 days in CM (+cytokines) relative to cultures incubated in CM only (–cytokines) for 14 days

$F_{1,7}$=302.57; P<0.001). Moreover, the overall cell counts of these converted 14-day-old cultures were similar to those seen in cultures incubated only 7 days in the differentiation media (compare TH-ir counts in Figs. 6 and 9).

Discussion

The numerous BrdU-ir cells observed in the fetal mesencephalic spheres in experiment 1 demonstrated that active proliferation was occurring in the cultures since this label can only be incorporated into actively dividing cells. Moreover, since the proliferating spheres utilized in that study were passaged at least 4 times and expanded 21 days prior to plating, it is unlikely that primary differentiated cells could have survived. These data suggest that the source of cells for all subsequent experiments was actively dividing cells which could be classified as either stem or progenitor cells. We previously demonstrated that IL-1 along with other cytokines induced the expression of TH-ir cells in E14.5 mesencephalic subependymal cultures. Given that IL-1 influences the differentiation of mesencephalic subependymal cells, it might be suggested that IL-1 would exert a similar effect on all subependymal cells. However, this does not appear to be the case, since IL-1 failed to induce conversion of E14.5 striatal subependymal cells into TH-ir cells in experiment 2. These data demonstrate that striatal subependymal and mesencephalic subependymal

cells respond differently to the same cytokine, and this finding is consistent with the notion that during fetal development, lineage restriction with respect to differentiation occurs along a rostral-caudal axis of the neural tube (Yamada et al. 1993; Placzek et al. 1993; Hynes et al. 1995a,b). In other words, since striatal and mesencephalic subependymal cells responded differently to the same environmental factor, i.e., IL-1, lineage restriction in these cells has already occurred. Therefore, subependymal cells isolated from the E14.5 striatum and mesencephalon should be classified as progenitor cells, and not omnipotent stem cells (Anderson 1989; McKay 1997; Ray et el. 1997; Stemple and Mahanthappa 1997). Since we have also demonstrated that the mesencephalic subependymal cells are self-renewing and pluripotent, these lineage-restricted mesencephalic subependymal cells meet the three major criteria for their classification as progenitor cells.

The influence of IL-1 on neuronal differentiation is plausible given that many hematopoietic cytokines including the interleukins are present in fetal brain parenchyma during neuronal maturation (Hynes et al. 1995a,b; Jessell and Dodd 1990; Kilpatrick and Bartlett 1993; McKay 1997; Temple 1989). The trophic effects of IL-1 on DA and other catecholaminergic neurons also have been documented both in in vitro and in vivo experiments (Akaneta et al. 1995; Nakao et al. 1994). Thus, similar to their effects on hematopoietic cell development, cytokines such as IL-1 likely play a role in CNS development and differentiation. However, we also included IL-11, LIF, and GDNF in our differentiation media because we previously showed that this mixture of cytokines increased numbers of converted TH-ir cells, and further these cells exhibited greater maturity in their neuronal morphology (Ling et al. 1998). In addition, freeze-fractured mesencephalic membrane fragments as well as media from striatal cultures were also included in the differentiation media. We included these because we have previously demonstrated that extracellular matrix proteins and an unknown trophic activity found in striatal conditioned media enhanced process development and cell body maturation in the converted DA neurons (Ling et al. 1998). Although the possibility cannot be completely ruled out that the mesencephalic membrane fragments or striatal conditioned media are critical to the conversion process, this seems unlikely since the addition of membrane fragments only enhanced the morphological development of cells when IL-1 was present (Ling et al. 1998), and we have previously shown that progenitor cells grown in co-culture with E14.5 striatal cultures failed to convert to DA neurons (Ptak et al. 1995). This suggests that the added cytokines, and in particular IL-1, play a central role in the conversion process. Although the relative contribution of each cytokine during conversion of mesencephalic progenitors into DA neurons is currently being evaluated in our laboratory, it is clear that IL-1 is a critical element since we have never observed significant numbers of TH-ir cells in progenitor cultures that did not contain IL-1. This might suggest that IL-1 induces the expression of specific receptors, which allow the progenitor cells to become responsive to the additional factors present in the differentiation media. For example, IL-1 may increase numbers of gp130-linked receptors, thereby increasing the progenitor cell response to LIF and IL-11 (Hirano et al. 1994). In addition, IL-1 may increase numbers of ret receptors, thereby increasing their response to GDNF (Durbec et al. 1996; Jing et al. 1996).

Regardless of the mechanism(s) involved, it is important to note that the relative percentage of TH-ir cells present in these cultures (~25%) was significantly higher than the 1–5% normally found in primary mesencephalic cultures. Since it has been demonstrated that progenitor cells differentiate into neurons and glia (Ray et al. 1997), the preferential enrichment of TH-ir cells in the converted cultures suggests that the differentiation media drive the conversion of progenitors into DA neurons. The studies with the Hu antibody presented here begin to address this issue.

The Hu antibody labels an RNA-binding protein found only in neurons and in progenitor cells destined to become neurons (Barami et al. 1995). It is expressed in neuronal precursors during their residence in the subependymal zone prior to migration and is considered the earliest known marker for the neuronal phenotype (Marusich and Weston 1992; Barami et al. 1995). In our mesencephalic progenitor spheres, a few Hu-ir cells were apparent within 3 h of plating in CM without cytokines (data not shown). It is possible the FCS-supplemented media very quickly induced expression of the neuronal phenotype or, alternately, the Hu-positive cells were present in the expansion cultures prior to plating. Unlike the round Hu-ir cells observed 3 h after plating, Hu-ir cells observed following 7 days incubation in CM (containing 10% FCS) exhibited a distinct neuronal morphology. This implicates FCS in neuronal maturation. However, incubation in the differentiation media increased numbers of Hu-ir cells in the cultures sixfold and suggests that the cytokines increased the number of progenitor cells which converted into neurons, approximately one-half of which were also TH-ir cells, as shown by the cell counts and the double-labeling studies. Thus, the differentiation media not only increased the number of progenitors converting into neurons, but also increased the number of neurons developing into DA neurons. As innumerable astrocytes were observed in the cultures, a ratio of Hu-ir to GFAP-ir cells could not be determined. Therefore, we could not establish if the differentiation media increased Hu-ir cells while decreasing GFAP-ir cells. This would have supported the notion that the differentiation media were "driving" the conversion of progenitors into neurons. However, it was clear that the differentiation media did increase the percentage of neurons converting into TH-ir cells, since these cell counts increased 58-fold relative to cultures incubated in CM (only), whereas the Hu-ir cell counts increased only sixfold. Thus, the differentiation media clearly increased the conversion rate of progenitor cells to TH-ir cells, and this highly increased conversion rate could not be accounted for by the observed preferential conversion of progenitors into Hu-ir cells.

Incubation in differentiation media appeared to increase the morphological maturation of neuronal cells in the mesencephalic progenitor cultures. While increased TH-ir cell counts were observed in cultures incubated in the differen-

tiation media, greater numbers of cells with processes were also observed. GAP43-ir cell counts were approximately 45% of the Hu-ir cell counts observed in sister cultures incubated with CM. Since GAP43 is considered a marker for neurons extending processes (Meiri et al. 1988), only about half of Hu-ir cells were likely to extend processes when the progenitor cells were incubated in CM with 10% FCS. However, addition of the differentiation media to sister cultures generated numbers of Hu-ir cells that were similar to numbers of GAP43-ir cells. This suggests that most of these neurons were extending processes. Although a similar study could not be performed on the astrocytes, it did appear that with incubation in CM, the astrocytes formed a confluent carpet with short thin ramifications. In contrast, with incubation in differentiation media, the astrocytes exhibited thick, dense ramifications. Thus, it appears that the differentiation media not only influenced the relative numbers of different cell types in progenitor cultures, it also influenced the morphological maturation of these cell types. It is possible that the mesencephalic membrane fragments included in the differentiation media were wholly responsible for these effects. Mesencephalic membrane fragments are thought to provide additional extracellular matrix proteins to the cultures, and these proteins may influence cell maturation (see Ling et al. 1998).

In experiment 4, we used double-labeling to characterize the converted TH-ir cells. While in cytokine-incubated progenitor cultures we observed numerous BrdU-ir cells that were also TH-ir, very few double-labeled cells were seen in sister cultures incubated in CM without cytokines. This suggests that the cytokine-enriched differentiation media converted mesencephalic progenitor cells into DA neurons. However, not all TH-ir cells were BrdU-ir, which might suggest that some of the TH-ir cells were not derived from a progenitor cell. Since the cultures were expanded for 21 days and passaged at least 4 times, it is highly unlikely that these cultures could have included primary neurons that survived the passaging process. In addition, as seen in Fig. 1, it is clear that the BrdU incubation procedure used did not label all the cells. This is often the case given the short duration of exposure to the label. Thus, it is quite possible that the TH-ir cells that did not double label for BrdU simply reflected that subpopulation of cells that did not originally incorporate the label. The double-labeling experiments also demonstrated that all TH-ir cells were also Hu-ir following exposure to differentiation media whereas cultures exposed to CM contained few and in most cases no cells with this double label. Based on these results we therefore conclude that the TH-ir cells seen in cultures exposed to media containing cytokines reflect a phenotypic conversion of progenitor cells into DA neurons.

A relevant issue is stability of the DA neuron phenotype once the cytokine-induced conversion of progenitor cells has occurred. Aside from the practical aspects, stability of the converted phenotype would lend insight into the mechanism underlying the conversion process. If the converted phenotype was dependent upon continuous exposure to cytokines, this would suggest the involvement of a translational event. However, if exposure to the differentiation media resulted in a more permanent expression of TH, transcriptional events are more likely involved. The results from experiment 5 demonstrated that 7 days following withdrawal of the cytokine-supplemented media, the TH-ir phenotype was still present. Moreover, the number of TH-ir cells present was similar to that seen immediately following 7 days incubation in the differentiation media. Thus, it is unlikely that the cytokines simply increased the levels of TH, thereby allowing detection by the immunoassay. Rather, it appears that elements in the differentiation media turned on the expression of TH through a transcriptional event. In addition, it appears that the various elements in the differentiation media were not responsible for maintaining the viability of the progenitor cells since the cell counts observed 7 days following differentiation media withdrawal were similar to those seen after 7 days exposure to the differentiation media. Thus, elements in the differentiation media primarily regulated the differentiation process. It was surprising to note, however, that the morphology of the cells in the cultures withdrawn from the differentiation media for 7 days was similar to that seen in the cultures after 7 days. Because of the presence of FCS in the CM used during the withdrawal phase of this experiment, we expected to see continued morphological development and process extension as is typically seen in primary mesencephalic cultures. The fact that this was not seen may be explained by the absence of trophic factors such as GDNF or, alternatively, that the cytokines used to convert these progenitor cells were not able to completely induce the typical DA neuron phenotype that is responsive to FCS. Be that as it may, the likely involvement of cytokines in a transcriptional event involved with neuronal differentiation has implications for our understanding of the relative roles of genetics and environment in the development of neuronal phenotypes.

Our data suggests an interplay between genetic predisposition and environmental factors. While elements in the differentiation media appeared to drive the conversion of progenitors into DA neurons, these cells exhibited a genetic predisposition to differentiate into neurons and glia. In fact, regardless of the source of the subependymal cells (striatal or mesencephalic), the "default" program appeared to be a combination of neurons and glia. However, none of the striatal progenitor cells converted into DA neurons by a differentiation media that did convert mesencephalic progenitors into DA neurons. This implicates a genetic predetermination consistent with a lineage restriction along the rostral-caudal axis of the neural tube (Hynes et al. 1995b). Such a predisposition cannot, however, be expressed without exposure to a specific environmental signal which, in the case of mesencephalic progenitor cells, appears to involve IL-1. Given the appropriate predisposition and environmental signal, mesencephalic progenitors can then become responsive to other environmental signals such as IL-11 or GDNF. Thus, it is a unique combination of predetermination and environment that results in the expression of the DA neuron. Several investigators have observed similar findings using other types of progenitor cells (see Bele et al. 1995; Birling and Price 1998; Shetty and Turner 1998) and Qian et al. (1997) have also shown that the genetic predisposition influencing the response to environmental signals changes with embryonic development age, implicating a

cascade of signaling events that regulate the expression of a neuronal phenotype. If this is indeed the case, then mesencephalic subependymal cells taken from early or older embryos might respond differently to environmental signals such as IL-1.

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Mesencephalic Progenitor Cells Converted to Dopamine Neurons by Hematopoietic Cytokines: A Source of Cells for Transplantation in Parkinson's Disease.

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Abstract:

Neural progenitor cells capable of division *in vitro* are potentially useful in providing a limitless, on-demand source of cells for grafting into patients with Parkinson's disease (PD) if the signals needed to control their conversion into dopamine (DA) neurons could be identified. In an effort to accomplish this, we clonally expanded rat mesencephalic progenitor cells and studied their response to hematopoietic cytokines which normally regulate cell phenotype and lineage restriction in bone marrow. Several of the clones responded to interleukin-1α (IL-1α) by expressing the DA neuron marker tyrosine hydroxylase (TH). The clone yielding the highest percentage of TH neurons was selected for further study and expressed additional characteristics of DA neurons including DA production, DA transporter, and expression of Nurr1 in response to combined exposure to IL-1α, IL-11, leukemia inhibitory factor, and glial cell line-derived neurotrophic factor. The response to cytokines was stable for several passages and after cryopreservation for several months. When grafted into the striatum of lesioned rats, these cells attenuated rotational behavior in the 6-hydroxydopamine rat model of PD to the same extent as freshly harvested ventral mesencephalic cells, although the rate of recovery was slower. These data demonstrate that mesencephalic progenitor cells can be expanded in culture to produce functional effects and suggest that hematopoietic cytokines participate in CNS development in a fashion analogous to their actions in bone marrow.

Key Words: Interleukins; Glial Cell Line-derived Neurotrophic Factor; leukemia inhibitory factor; 6-hydroxydopamine; Nurr1; Tissue Culture; Clonal expansion.

Introduction:

Transplantation of embryonic ventral mesencephalic cells into the striatum of patients with advanced Parkinson's disease (PD) has emerged as a viable treatment (Olanow et al., 1996). Although grafted cells have been shown to survive and significantly attenuate symptoms of PD (Kordower et al., 1996, 1997a; Hagell et al., 1999; Ross 1999; Lindvall et al., 1998), the logistics of procuring adequate amounts of embryonic tissue, screening it for pathogens, and implanting it promptly, severely limits the wider utility of this procedure. Moreover, the use of embryonic tissue poses ethical concerns to many (Hardy 1996; Fletcher 1992). Other tissue sources such as porcine ventral mesencephalon (Galpern et al., 1996; Molenaar et al., 1997), genetically transformed cells (Barkats et al., 1998; Lindvall 1999), and dopamine (DA) neuron precursor/progenitor cells (Wilby et al., 1999; Wagner et al., 1999; Svendsen et al., 1997) have been investigated as alternatives. In particular, the use of mesencephalic progenitor cells as a tissue source has significant advantages as they readily replicate providing an almost unlimited source of cells with fewer ethical issues. However, identifying the signals needed to reliably convert mesencephalic progenitor cells into DA neurons has proven elusive.

Hematopoietic cytokines are known to participate in the differentiation of hematopoietic stem cells in bone marrow and an emerging literature suggests that they also are present in developing brain (Feuerstein et al., 1998; Zhao and Schwartz 1998). We previously demonstrated that mesencephalic progenitor cell could differentiate into DA neuron phenotype in response to signals provided by soluble (cytokines, GDNF, and striatal conditioned media) and insoluble (mesencephalic membrane fragments) molecules (Ling et al., 1998). Differentiated cells were immunoreactive for tyrosine hydroxylase (TH) as well as several other DA neuron markers (Potter et al., 1999). Hematopoietic cytokines thus appear to perform analogous functions in multiple organs and may represent key signaling molecules in CNS development.

Although cytokine-induced conversion of mesencephalic precursor cells to DA neurons is

encouraging, several issues remain before advocating their potential use for grafting, including: 1) reliability of conversion rate; 2) ability to cryopreserve these cells; and 3) ability to survive and maintain a stable phenotype *in vivo*. Mesencephalic progenitor cells that were not clonally expanded revealed variability in the DA neuron conversion rates from 2% to 40% in response to cytokines. Since the intent of using progenitor cells is to provide a reliable cell source for grafting, such variability would be unacceptable. Progenitor cells in general also appear to have a limited expansion period and spontaneous differentiation *in vitro*, it would be helpful to determine if these cells were able to survive cryopreservation so that cells from an early passage could later be thawed and expanded. Finally, demonstrating conversion of progenitor cells into DA neurons in culture does not necessarily imply that the cells will remain functional once grafted. In an effort to address these issues, we performed a clonal expansion study of mesencephalic progenitor cells, froze them in di-methyl sulfoxide, and grafted them into striatum of the 6-hydroxydopamine lesioned rats – an animal model commonly used to study grafting strategies for PD.

Methods and Materials:

Animals: Progenitor cells were harvested from timed, gravid E14.5 Sprague Dawley rats (Zivic Miller, Zelienople, PA). Transplantation studies were performed on four month old Sprague Dawley rats. All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility that were temperature and humidity controlled with lights on between 0600 and 1800 h. All animal procedures were approved by the Rush-Presbyterian-St. Luke's Medical Center Institutional Animal Care and Utilization Committee prior to the study.

Embryonic Tissue Harvests: The ventral mesencephalon (VM) was harvested from E14.5 Sprague Dawley rats under deep pentobarbital sedation (40 mg/kg i.p) as previously described (Ptak et al., 1995). Pooled VM was incubated in 0.1% trypsin for 30 min, incubated in DNase (40 μg /ml) for 10 min at 37°C, and gently triturated into a cell suspension using a fire-polished pipette. Cells were counted using a hemacytometer. Cells used for control VM transplants were immediately grafted

into host rats. Cells destined for cloning, were plated onto 25 cm^2 flasks (400,000 trypan blue excluding cells/ml) in 5 ml of expansion media (Hamm's F12/DMEM 1:3; 100 units penicillin/100μg streptomycin [P/S]/ml; 2 ml B27 [1:50; Gibco] per 100 ml media with 20 ng/ml epidermal growth factor (EGF)). The cells were left undisturbed in a humidified incubator (37°C and 5% CO_2) for one week. The yield from this procedure was approximately 50-100 "proliferation spheres" per cm^2.

Clonal Expansion: After establishing the primary cultures, cells were passaged after 10 days. The limiting dilutions techniques was then used to perform a clonal expansion (Unsicker et al., 1992). Five hundred cells from the first passage were plated at 0.1, 0.5, or 1 cell per well in 96-well Costar plates containing expansion media. Twenty-four hours later, wells containing only one cell were labeled and fed with fresh expansion media every six days. Of the 160 single cells identified, twenty-four clones survived and were passaged individually three additional times. The surviving clones were expanded to yield adequate numbers of cells for plating. Once the number of cells in each clone reached 1 X 10^6 or more, the cells were plated onto poly-l-lysine coated 48-well plates with and without IL-1α (100 pg/ml) in differentiation media (10% fetal calf serum (FCS), 44.5% F-12, 44.5% DMEM, 100 units penicillin/ml, and 100 μg streptomycin/ml). A portion of these clones were exposed to IL-1α or media without IL-1α for five days. The cultures were subsequently fixed, stained for tyrosine hydroxylase (TH), and the percentage of TH immunoreactive (THir) cells was assessed as described below. The remaining cells were maintained in the expansion media until reaching 10 X 10^6 or more and passaged every six days.

Culture Immunocytochemistry: Numerous immunocytochemical stains were performed on the cultures. In general, cultures were fixed with 3.7% paraformaldedyde solution for 20 minutes and washed with phosphate buffered saline (PBS). Cultures were then incubated with 3% normal horse serum in PBS with 0.25% Triton X and 0.001% H_2O_2 for 1 hour followed by overnight incubation with 1:5,000 diluted TH primary antibody (mouse IgG from Incstar; Stillwater, MN) at 4°C. On the second day, the plates were washed with PBS and incubated with biotinylated horse anti-mouse IgG

for 1 hour at room temperature. The plates were washed and reacted with avidin-biotin complex (ABC, Vector Laboratories, CA) for 1 hour. Plates then were sequentially washed with PBS and imidazole/sodium acetate solutions. The TH immunoreactive cells were visualized using the subtract solution (nickel sulfate 0.25%, 3,3'-diaminobenzidine 0.05%, and H_2O_2 0.005%). The plates were washed and stored in PBS for THir cell counting. For assessment of other markers, the antibodies used for these procedures were rabbit anti-dopa decarboxylase (DDD; 1:2000; Chemicom, Temecula CA), rat anti-DA transporter (DAT; 1:5000; Chemicom, Temecula CA), mouse anti-nestin (1:5000; Chemicom, Temecula CA), mouse anti-DA (1:500; Fitzgerald Int. Inc. Concord MA), and mouse anti-glial fibrillary acidic protein (GFAP; 1:5000; Sigma, St. Louis MO). The procedures were similar to the TH immunocytochemistry procedure except different primary and secondary antibodies were used. All secondary antibodies were biotinylated (anti-mouse IgG, anti-rabbit IgG, or anti-rat IgG).

Con-focal microscopy staining immunocytochemical co-localization for Nurr1 with TH, glial fibrillary acidic protein, or microtubule associated protein (MAP-2) was accomplished using two antibodies from two different species. The primary anti-TH, anti-Map-2 (Calbiocan), and anti-GFAP were monoclonal mouse IgG and the anti-Nurr1 was polyclonal rabbit IgG (Santa Cruz, CA). The secondary antibody to anti-TH or anti-MAP-2 IgG was Texas Red conjugated goat anti-mouse IgG (Vector, Burlingame, MN). The Nurr1 protein was visualized by using anti-Nurr1, biotinylated secondary antibody, and fluorescein conjugated streptavidin. The stained cultures were analyzed using the Olympus Fluoview confocal microscope.

Nurr1 Western Blot: Mesencephalic cells in the culture plates were washed with cold PBS and then 100 µl lysis buffer (0.1M Tris buffered saline, pH 8.1, containing 1 mM EDTA, 1% aprotinin, 10 µg/ml leupeptin, 14 µg/ml pepstatin, 4 mM PMSF, and 0.5% NP-40) was added to each well. Plates were gently shaken at 4 °C for 4 hours and then the lysate was harvested. Collected lysate was spun down in micro tubes to remove cellular debris. All the lysate was then centrifuged in Millipore Ultrafree-MC centrifugal filtration units to remove extra PN-40 and to concentrate the remaining

protein. Equal amounts of protein were loaded under reducing conditions onto a 10% SDS gel. After electrophoresis, the protein was blotted onto nitrocellulose membrane. The membrane was washed with TBS and blocked with 20% fat free milk in TBS. The membrane was then incubated with anti-Nurr1 antibody (1:1000, Santa Cruz, CA) overnight at 4 °C. After washing, the membrane was incubated with biotinylated secondary antibody followed by peroxidase conjugated avidin-biotin complex (ABC). The bands were visualized by using 3,3'-diaminobenzidine substrate. The membrane was finally washed for photography.

Cell Count Assessment in Culture: The number of THir cells in the clonally expanded cultures was assessed by an individual blinded to treatment history. The number of cells was assessed by counting the cells in 10 random fields of the cultures at 10X40 magnification using bright field. In many cases the THir cells were clustered as is common in neurosphere studies such as these where incomplete trituration is the rule since we have observed that these cells do not tolerate extensive trituration (Z.D. Ling, unpublished observation). Thus, the cell count data represents an approximation. The total number of cells (dark stained THir cells and un-stained cells) was counted in the fields and the number of THir cells/total cells was used as an index of THir conversion rate.

Cryopreservation of Clones: The expanded progenitor cell clones were centrifuged at 100g for 2 minutes to remove old culture media. The pellets were re-suspended in ice-cold DMEM/F12 media containing 2% B27 supplement, 10% bovine serum albumin (BSA) and EGF (100 ng/ml). An equal volume of cold DMEM/F12 media containing 20% dimethyl sulphoxide (DMSO) was then added drop-by-drop. Cells were mixed well with media and aliquoted into Nunc cryotubes at approximately $5X10^6$ cells per tube. Tubes were placed at -80°C overnight and transferred into a liquid nitrogen tank the next day. When thawing was performed, a tube was taken out from liquid nitrogen and immediately submerged into 37°C clean water for approximately 2 minutes. Cells were then transferred into a 15 ml tube and washed once with pre-warmed expansion media (DMEM/F12, EGF (20 ng/ml), 2% B27 supplement, and 1% pen/strep). Finally, cells were cultured in 5 ml expansion

media until they were ready for passaging.

Preparation of Cells for Transplantation: In order to determine the optimum exposure time to differentiation media for grafting, the progenitor cells were cultured in differentiation media in un-coated tissue culture tubes at 37°C. The differentiation media consisted of FCS, (10%),P/S; IL-1α, (100 pg/ml); IL-11, (1 ng/ml); LIF, (1 ng/ml), and GDNF, (10 ng/ml) in 45%DMEM:45% Hamm's F12. At varying time points (1, 2, 3, 4, 5, 6, 7, and 8 hours after incubation, data not shown) the differentiation media was washed off and the cells were plated onto PLL-coated 48-well plates. After 6 days in complete media without cytokines, the cultures were fixed and stained as described above. Five hours exposure to differentiation media was the shortest time determined to induce stable THir cell conversion and was used for all of the remaining studies.

6-hydroxydopamine (6OHDA) Lesioning and Grafting Procedure: Young (4 month old) adult male Sprague-Dawley rats received stereotaxic surgery for the induction of unilateral mesostriatal DA depletion via injection of 6OHDA (RBI, Natick, MA, 5μg/μl in 0.2% ascorbic acid-saline vehicle), with 2μl infused into each of two sites: -4.3/+1.2/-7.5 and -4.8/+1.7/-7.5 (Paxinos and Watson 1996). The functional response to the lesion was tested using amphetamine-induced (5mg/kg, i.p.) rotational behavior in a computer-assisted rotometer system two and four weeks after lesioning. Unilaterally lesioned rats have asymmetric nigro-striatal DA projections that rotate toward the side of the in response to amphetamine. The rotational rate is widely used as an index of that asymmetry.

Grafting Study: Those rats achieving rotational criteria (≥ 5 rotations/min) indicative of > 90% unilateral striatal DA depletion were assigned to one of four treatment groups: 1) freshly harvested ventral mesencephalic cell grafts (VM; n=7); 2) cytokine-converted progenitor cells (Cyt. Prog.;n=7); 3)non-converted progenitor cells (NCyt. Prog; n=6;cells incubated in media without cytokines); and 4) sham controls (Sham; n=5). Implants were 70,000 cells/1μl, with 3μl implanted into each of 2 sites in the denervated caudate nucleus (coordinates: +1.0/+2.5/-5/0; -0.6/+4.2/-5.8). While we attempted

to equalize the number of cells implanted across experimental groups, estimation of cell numbers in the progenitor cell groups was problematic due to their tendency to aggregate. Thus, it is likely that more cells were implanted in progenitor cell groups than in the fetal VM implant group. Rats were tested for amphetamine-induced rotational behavior again 2, 4, 6, and 8 weeks after grafting.

Grafted Brain Assessment: Twenty four hours following the conclusion of behavioral testing, animals were deeply anesthetized (50 mg/kg pentobarbital) and given intracardiac perfusion with physiological saline followed by 4% buffered paraformaldehyde in a 0.1 M phosphate buffer. Histological evaluation of surviving, grafted DA neurons was performed as described previously (Collier et al., 1999). Each brain was removed, postfixed for 24 hours, and immersed in 30% sucrose in 0.1M phosphate buffer. Fixed brains were sliced on a freezing microtome (35 μm) and free-floating brain sections were immunostained by using antisera directed against TH (1:4000, Chemicon, Temecula, CA, overnight at 25°C). Triton X-100 (0.3%) was added to the Tris buffer during incubations and rinses to permeabilize cell and nuclear membranes. After Tris rinses, cells were incubated in biotin conjugated goat anti mouse IgG secondary antibody (1:400, Chemicon) for 1 hour, rinsed again, and incubated in ABC-peroxidase reagent (Vector, Burlingame, CA) for 1 hour. Following rinses, antisera labeling was visualized by exposure to 0.5 mg/ml 3,3' diaminobenzidine (DAB) and 0.03% H2O2 in Tris buffer. Relative survival of grafted DA neurons was estimated by counting TH-positive neurons in the 2 graft sites of each animal at 210μm intervals throughout each graft. Cells counted were required to be TH-positive and have either a visible nucleus or at least one neurite of length greater than 2X the diameter of the cell soma.

Statistics: Statistical changes in rotational behavior were analyzed with repeated measures ANOVA followed by Fisher's PLSD and means comparisons tests to identify specific differences among groups. Cell counts were analyzed with factorial ANOVA followed by Fisher's PLSD test.

Results:

Clonal Expansion: The number of TH immunoreactive (ir) cells in the cultures of the expanded clones was highly variable although all of the cultures containing IL-1α had at least a few

immunoreactive THir cells (Table I) with several of the cultures expressing predominantly THir cells (Fig. 1a). Many of the THir cells in these cultures did not exhibit traditional DA neuron morphology, however. Many of the cell bodies were small and not highly developed while many processes were tapered and did not contain varicosities typical of DA neurons in culture. In expanded cultures of those clones incubated with IL-1α that did not exhibit significant THir conversion (Fig. 1b), numerous clusters of GFAPir cells were seen (Fig. 1c) indicating that clonal expansion of progenitor cells from the same brain region yield different cell types. Those clones incubated in media without IL-1α did not contain any THir cells.

One of the clones containing the greatest percentage of THir cells (MPC-C9) following incubation with IL-1α was passaged five more times to generate adequate tissue for further study. Several hundred thousand cells from this clone were also washed and cryopreserved in media for future study.

Nurr1 Studies in Culture: The passaged cells from clone MPC-C9 that had not been cryopreserved were exposed to media containing IL-1α, IL-11, LIF, and GDNF (Ling et al., 1998) or control media for six days in culture and then examined immunocytochemically for TH, MAO-2, GFAP, and Nurr1. Nurr1 is a member of the superfamily of nuclear transcription factors and is considered essential for induction of the DA neuron phenotype as Nurr1-null mutant mice fail to develop DA neurons (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998). It is thus considered a marker for DA neurons. In control cultures, where no cytokines were added, Nurr1 was expressed at a very low level (Fig. 2A) and TH was undetectable (Fig. 2B). In contrast, the clone exposed to cytokines expressed both phenotypic markers (Fig. 2E and 2F, respectively). All THir cells were immunoreactive for Nurr1 while many Nurr1ir cells did not react for TH (Fig. 2G). However, when the cultures were evaluated for the non-specific marker for neurons (MAP-2) and Nurr1, all the neurons expressed both phenotypes (Figure 2H). This finding is consistent with the report that Nurr1 is thought to be an essential transcription factor in neuronal development in general and not limited only to the DA neuron phenotype (Xiao et al., 1996; Xing et al., 1997).

A similar study was performed on MPC-C9 to assess GFAP and Nurr1 co-localization (Figure 3). The few GFAPir cell clusters that were seen in MPC-C9 were generally located in the fringes of the cell clusters. Unlike TH, GFAPir cells were present in the cultures exposed to both the cytokine

free media and the cytokine mixture (Figure 3B and E). However, confocal microscopy demonstrated that Nurr1 and GFAP were not co-localized (Figure 3C and F).

The apparent up-regulation of Nurr1 by the cytokine mixture was verified using Western Blot (Figure 4). The protein from the cell lysates from cultures exposed to media only (Figure 4A), IL-1α only (Figure 4B), and the complete cytokine mixture (Figure 4C) were assessed for Nurr1 (Figure 4). As was apparent in the con-focal imagery, a low level of Nurr1 was detected using Western blot in the cultures exposed to media. In parallel with the con-focal images addition of IL-1α, or the full cytokine mixture increased Nurr1 immunofluorescence as well as the protein.

Cryopreservation Studies: The MPC-C9 cells that were cryopreserved were thawed after four months, expanded using EGF, passaged several times, and studied for phenotypic markers of DA neurons (Potter 1999) in response to the full cytokine mixture. Although they were frozen, thawed, and subsequently passaged, expanded cells of the MPC-C9 clone still expressed phenotypic markers characteristic of DA neurons including TH (Fig. 5A), dopa-decarboxylase (Fig. 5B) DA (Fig. 5C), and the DA transporter (Fig. 5D) following incubation in differentiation media containing cytokines. The morphologic appearance of the these cells was comparable with our previous observations using non-cloned cells where IL-11, LIF, GDNF as well as IL-1α were required to induce a morphology typical of normal cultured DA neurons. Thus, unlike the immature cells seen in culture following incubation with only IL-1α, cultures incubated with the complete cytokine mixture contained THir cells with fully developed morphologies including large somas, non-tapered processes, and long processes containing numerous varicosities. However, the percentage of cells converted to THir neurons following cryopreservation was not as pronounced as that seen when MPC-C9 was first clonally expanded where approximately 98% of the cells were THir. Thus, the percentage of THir cells seen in the cultures following cryopreservation was estimated at 72.66% ± 8.51 which was similar to the number of DATir (63.33% ± 4.04), DAir (65.66% ± 7.23), and DDCir (72.6% ± 10.78) cells seen in sister cultures. Following cryopreservation a greater number of cells immunoreactive for the astrocyte marker glial-fibrillary acidic protein (GFAP) were seen (24.00% ±3.60; Fig. 5E). Cells immunoreactive for the stem cell marker nestin (13.28 %± 2.00; Fig. 5F) were still seen in the cultures suggesting that cytokine-induced conversion to a committed phenotype does not preclude regeneration of the progenitor cell. It also is important to note that cultures that exhibited conversion to THir cells or GFAP cells maintained the tendency to convert to the same phenotype

in response to the cytokine mixture. Thus, when studied on different occasions, MPC-9 would always predominantly convert to THir cells while MPC-4 converted predominantly to GFAPir enriched cultures.

Grafting Study: Although the characteristics of the converted progenitor cells in tissue culture were strikingly similar to typical primary DA neurons, the utility of these cells as a source of tissue for neural grafting can best be assessed *in vivo*. MPC-C9 was therefore subsequently tested in the rat unilateral 6-hydroxydopamine (6OHDA) lesion model of PD. Following a five hour exposure to the differentiation media containing cytokines, the cells were grafted (Cyt. Prog.). These grafts were compared with animals grafted with freshly harvested ventral mesencephalic cells (VM), progenitor cells exposed to differentiation media without cytokines (NCyt. Prog.), and sham controls. It is important to note that the cell viability observed during the grafting procedure differed among the groups. Following the completion of grafting the cell viability of the freshly harvested cells was 99.37% whereas the viability in the Cyt.Prog. cells was down to 60.02%.

Animals which received freshly harvested ventral mesencephalic cells exhibited a consistent reduction in their amphetamine-induced rotation rates during the eight weeks following grafting (Fig. 6A). Repeated measures ANOVA revealed significant differences between groups ($F_{3,21}$=7.46, p=0.0014), over time after grafting ($F_{4,84}$=14.28, p<0.0001), and in the group X time interaction ($F_{12,84}$=7.21, p<0.0001). A similar pattern of rotational rate change, albeit delayed by two weeks, was observed in the animals grafted with the cytokine-exposed clones. In contrast, animals grafted with the clones that were exposed to media without cytokines, as well as the animals exposed to sham surgery, failed to alter their rotational rates following grafting. Following perfusion-fixation, the grafted striata from the animals were analyzed immunocytochemically for TH. Only those animals that had received freshly harvested ventral mesencephalic cells (VM) or cytokine-exposed clones (Cyt. Prog.)exhibited THir cells in their grafted striata (Fig. 6B). Grafts of freshly harvested cells yielded approximately three-fold more THir cells than those animals grafted with cytokine-exposed clones ($F_{2,17}$= 38.90; p < 0.001). Reinnervation of the striatum was commensurate with relative cell survival as converted mesencephalic progenitor cells provided innervation that was not as pronounced (Fig. 6D) as that seen in the animals grafted with fresh VM (Fig. 6C), but was in clear contrast to the near-total absence of striatal THir fibers seen in the animals grafted with mesencephalic progenitor cells not exposed to cytokines (Fig. 6E).

Discussion:

IL-1α, IL-11, LIF, and the neurotrophin GDNF were demonstrated to participate in the induction of the DA neuron phenotype from mesencephalic progenitor cells. One of the problems we encountered with these cultures was a variable conversion rate. Thus, following incubation in the cytokine solution, THir cell counts would vary between 2% and 40% although the conversion rate was generally around 25%. Despite this variation, the observed conversion rate was significant since primary mesencephalic cultures generally exhibit only 0.5-1% DA neurons although some groups have achieved 20% enrichment using specialized dissection protocols (Takeshima et al., 1996). This disparity suggested a primary role for hematopoietic cytokines, and in particular IL-1α, in the phenotype induction process. This was further verified when it was shown that IL-1α turned on the expression of the gp130 receptor which mediates the effects of IL-11 and LIF. Thus, although both IL-11 and LIF participated in the full maturation of the DA phenotype when included in the cytokine mixture, neither were effective at inducing expression of TH in the absence of IL-1α. If IL-1α was indeed a primary inducer of the DA phenotype, then the observed variability in the conversion rate suggested variability in the progenitor cell population. We thus set out to perform a clonal expansion to test this hypothesis.

The results from the clonal expansion study similarly yielded significant conversion variation in response to IL-1α. Thus, of the successfully cloned cells, the number of THir cells observed following incubation with IL-1α varied between 2 and 98% supporting the hypothesis that within the subventricular region of the mesencephalon, progenitor cells in different degrees of lineage restriction are present. Because of the existence of different subsets of progenitor cells, the percentage of progenitors converted by IL-1α in our previous studies where non-cloned progenitors were studied would have been dependent upon the mix of progenitors collected in a particular dissection. When the MPC-C9 clone was passaged three additional times for further study, the THir cell conversion rate in response to the cytokine mixture was approximately 75%. The disparity between the very high conversion rate seen during the initial screening and the 75% conversion rate seen following passage likely reflects our previous observation that IL-1α alone is not capable of inducing the full neuronal phenotype (Ling et al., 1998) or that non-dopaminergic as well as DA neurons can transiently express markers for DA neurons (Mons et al., 1989). It is also important to appreciate that the percentage conversion rates were estimates because it was impossible to perform accurate

cell counts in the clusters. However, the stability of the phenotype conversion rate of ~75% was maintained even after cryopreservation for several months suggesting that the MPC-C9 clone was stable in its response to cytokines.

Progenitor cells in the mesencephalon of E14.5 rat embryos are likely analogous to the cells of the subventricular zone first described by Reynolds and Weiss (1992). This layer of tissue extends throughout the nervous system from the rostral regions of the brain where it is found inferior to the lateral ventricles down into the spinal cord where it is adjacent to the central canal (Gates et al., 1995; Alonso 1999; Liu et al., 1999). Stem cells have also been isolated from the subgranular zone of the dentate gyrus in the hippocampus as well as in the retina (Suhonen et al., 1996; Reh and Levine 1998). Although controversy surrounds the exact cell type involved (Kendler and Golden 1996), there is consensus that the subventricular zone is a primordial region from which most cells of the brain develop, and further, that this layer remains active and capable of forming new cells into adulthood. Whether or not these cells play a role in tissue repair in the adult brain is currently unknown, but numerous laboratories have shown that these cells exist in the developing and adult brain and that they can be isolated and expanded in tissue culture (Gates et al., 1995; Alonso 1999; Garcia-Verdugo et al., 1998). In order for these cells to be considered stem or progenitor cells, they must meet certain criteria including the ability to self-replicate and develop into more than one cell type (Levison and Goldman 1997). The mesencephalic progenitor cells studied her meet these criteria. Thus, in the presence of EGF they were able to divide readily. Moreover, they were immunoreactive for nestin, a traditional cell marker for progenitor cells. In addition, following exposure to the cytokine mixture used here, they developed into at least three different cell types including DA neurons, non-dopaminergic MAP-2 immunoreactive cells, and GFAP immunoreactive cells. Whether or not these precursor cells can divide indefinitely is unknown at this time and studies are under way to study this characteristic. However, we have previously reported that the passaging time needed for the non-cloned progenitor cells to expand began to slow after 5 months (Ling et al., 1998). Whether this is true for the MPC-C9 clone studied here remains to be established. If indeed these cells have a limited self-renewal, they are probably best classified as committed neural progenitor cells since current thinking suggests that stem cells have an unlimited ability to self-replicate (Anderson 1989; McKay 1997).

The notion that different types of precursor cells can exist within the subventricular zone of

a given region of the brain is consistent with the view that cells progress from stem cells to committed phenotypes by progressive lineage restriction (Yamada et al., 1993; Placzek et al., 1993; Hynes et al., 1995a). Recent studies have shown that stem cells respond to signals in the local environment which determine phenotype commitment (et al., 1995b). However, it is also likely that environmental cues, even within a small region of the brain like the mesencephalon, will generate a pool of cells with various degrees of lineage restriction that are likely to become progressively more responsive to one cue and less affected by another. Incubation of the various clones with IL-1α selected those cells responsive to this cytokine while other precursors in the pool were significantly less responsive (i.e., MPC-C4). Indeed, our strategy from the outset of this project was to attempt to identify precursor cells committed to the DA phenotype based on the assumption that it would be easier to identify the one or two signals needed to control their final conversion to this phenotype rather than attempt to identify all the elements of the cascade needed to convert an embryonic stem cell to a DA neuron.

We previously demonstrated that mesencephalic progenitor cells were immunoreactive for TH, DA, dopa decarboxylase, and the DA transporter (Ling et al., 1998). The same four phenotype markers were detected in the MPC-C9 clone, even after cryopreservation. Incubating the clone in the cytokine mixture also dramatically up-regulated the expression of Nurr1. As a nuclear transcription factor, Nurr1 is permissive for induction of the DA neuron phenotype (Saucedo-Cardenas et al., 1998). Although it is found in several other neuron types, it is expressed in all DA neurons found in the mesencephalon. Incubation of MPC-C9 with the cytokine mixture increased Nurr1 expression from a very low level (albeit apparently cellular) to a very high level that coincided with TH expression. This observation was confirmed using the Western blot procedure. All THir cells were immunoreactive for Nurr1, but there were also numerous Nurr1 immunoreactive cells that were not THir. When Nurr1 and the non-specific neuron marker MAP-2 were studied, it was clear that all MAP-2ir cells were also Nurr1 immunoreactive. This was not, however, the case for GFAP. Thus, astrocytes were present in the cultures in the presence and the absence of the cytokines and although it was clear that the cytokine mixture up-regulated the production of Nurr1 in these cultures, GFAP and Nurr1 were not co-localized. This further reinforces the notion that Nurr1 is a transcription factor for neurons.

Although exposure to cytokines appears to reliably induce numerous markers of the DA neuron phenotype in tissue culture, whether or not these cells are functional was not known. In

addition, we previously demonstrated that exposure to the cytokine mixture was able to up-regulate the expression of TH and TH was still detected in the cultures seven days following withdrawal of the cytokines (Potter et al., 1999). However, whether or not the DA phenotype would be maintained in vivo was in question. We therefore grafted the cells into rats with 6OHDA lesions to not only assesses functionality, but maintenance of phenotypic expression in brain parenchyma. The results from this study affirmed both questions.

Both the freshly harvested and cytokine-exposed cells produced significant behavioral effects on rotational asymmetry. This result is consistent with the number of cells detected in the graft site. Grafts of freshly harvested mesencephalic cells possessed an average of 1,386.84 ± 173.38 THir cells per graft, as estimated by the method of Abercrombie (1946). Grafts of cytokine-exposed clones contained, on average, 364.77 ± 74.34 THir cells per graft. Therefore, both graft types are comprised of adequate numbers of THir neurons to produce significant functional effects in the rotation test (Brundin et al., 1985).

Amphetamine-induced rotation in the unilateral 6OHDA lesioned rat reflects asymmetric release of DA from DA terminals in the striatum (Zetterstrom et al., 1986). The attenuated rotational response observed in the animals grafted with both freshly harvested ventral mesencephalic cells and cytokine-exposed clones indicated that functional DA release was taking place in the ipsilateral striatum, indicative of surviving DA neurons. This suggests that a brief exposure to the cytokines induced a stable conversion of the progenitor cells into DA neurons that remained functional following grafting. Although the cytokine exposed progenitor clone attenuated rotation in the animals, the rate or attenuation was delayed by two weeks relative to animals grafted with freshly harvested ventral mesencephalic tissue. This delay is consistent with the number of cells detected in the graft site. Thus, the greater the number of DA cells present in a graft site, the more rapid the rate of recovery from rotational asymmetry (Collier et al., 1999). It can thus be presumed that the delay in rotation attenuation in the animals grafted with cytokine exposed cells was due to the lower survival rate of these cells. The reason for this reduced survival rate is currently unknown and may be a consequence of several factors.

We have observed that the mesencephalic progenitor cells are much more sensitive to trituration than normal VM cells which could account for the lower survival of the former since the

cells require trituration as part of the grafting procedure used here. The progenitor cells tend to form tight clusters and mechanical trituration sufficient to completely dissociate these cells generally kills all the cells in the cultures. Why these cells would have this response is unknown at this time. In addition, we observed that during the 4 hour grafting procedure, the viability of the progenitor cells dropped from greater that 90% to 60% as assessed by exclusion of trypan blue. Analysis of the individual animals revealed that those rats grafted with cytokines exposed progenitors early in the day had higher cell counts and a more rapid attenuation of their rotational asymmetry. This suggests that one of the contributing factors to the delayed rotational attenuation and reduced survival rate was cell death in the incubation media prior to grafting. This is in contrast to the stable viability seen with freshly harvested VM cells where the cell viability at the end of the grafting day was >90%. Developing a strategy aimed at augmenting survival of grafted progenitors and maintaining phenotype expression is an ongoing focus of our work.

Taken together these data demonstrate that E14.5 mesencephalic progenitor cells can be induced to convert into functional DA neurons by hematopoietic cytokines. Unlike other strategies where the cells used as a potential source for DA neuron grafting have been genetically modified or derived from tumor cell lines, these progenitor cells may represent a more "natural" and safer alternative source of cells for grafting. Moreover, since they possess numerous phenotypic markers for DA neurons, hematopoietic cytokines may represent elements of the final signal transduction pathway naturally occurring in the ventral mesencephalon. Given that a similar conversion can be made using human tissue, these cells may be useful in providing a virtually unlimited supply of on-demand cells for grafting in the treatment of PD. The data further suggest that hematopoietic cytokines participate in the control and regulation of neuronal phenotype within the developing nervous system in general and that other neuronal progenitor cells may be induced to convert to specific phenotypes following exposure to other specific hematopoietic cytokines (Potter et al., 1999).

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Figure Legends:

Figure 1: Reverse-phase photomicrograph of clone MPC-C9 (A) and MPC-C4 (B) following exposure to IL-1α and immunochemical assessment for TH. In A note that cells in the clone have extended very long processes (toward the upper left) from the central core of the neurosphere (bottom right) and that almost all of the cells were immunoreactive for TH. Many of the cells exhibit atypical DA neuron soma morphology (arrows) with processes that taper. In contrast to the robust, healthy clone seen in A, many of the clones exposed to IL-1α, such as that depicted in B, were small, contained few THir cells and had a "glia-like" morphology. Another neurosphere from MPC-C4 was immunochemically assessed for glial fibrillary acidic protein (GFAP), a marker used to identify type II astrocytes. The vast majority of cells were GFAPir. (Magnification bar = 20 μm).

Figure 2: Confocal images of MPC-C9 exposed to control media (A-D) or media containing IL-1α, IL-11, LIF, and GDNF (E-H). After exposure to the media for 5 days, the clones were stained for Nurr1 (green immunofluorescence) and TH (red immunofluorescence). Cells immunoreacting to both antibodies are yellow in the con-focal image. Note the low level of Nurr1 immunoreactivity in the clones exposed to control media (A and C) and the dramatic increase in expression seen following exposure to cytokines (E and G). Moreover, in the absence of cytokine exposure, no THir cells were observed (B). In marked contrast, exposure to cytokines dramatically increased expression of TH (F) and all the THir cells also reacted positively for Nurr1 (G). However, numerous cells were immunoreactive only to Nurr1 (green cells in G). When the same clone was evaluated for Nurr1 immunoreactivity and its co-localization with the non-specific neuron marker microtubule associated protein (MAP-2) in response to control media (D) or the cytokine media (H), all of the MAP-2ir cells were also immunoreactive for Nurr1. (Magnification bar = 40 μm)

Figure 3. Confocal images of MPC-C9 exposed to control media (A-C) or media containing IL-1α, IL-11, LIF, and GDNF (D-F). After exposure to the media for 5 days, the clones were stained for Nurr1 (green immunofluorescence) and GFAP (red immunofluorescence). Cells immunoreacting to both antibodies would show up yellow in the con-focal image. In the periphery of the main neurosphere where the majority of the GFAP cells are usually found in this clone a small cluster of GFAPir are seen in both the control cells (B) and the cultures exposed to the cytokine mixture (E).

As was seen in Figure 2, exposure to the cytokine mixture increased the expression of Nurr1ir (compare A with D) although the response was not as robust as that seen in Figure 2. However, exposure to neither media led to GFAP and Nurr1 co-localization © or F). (Magnification bar =20 μm).

Figure 4. Confocal image of MPC-C9 exposed to control media (A), media containing IL-1α only (B) or , IL-1α, IL-11, LIF, and GDNF (C). After exposure to the media for 3 days, the clones were stained for Nurr1 (green immunofluorescence). The apparent amount of fluorescence was increased following incubation with IL-1α, and increased further following exposure to the full cytokine mixture. This apparent increase was confirmed using Western blot (D) where lanes a, b, anc c reflect the amount of protein in the lysates from the respective cultures. Nurr1 protein migrated between 97 Kd and 45 Kd molecular standards (mw. st.).

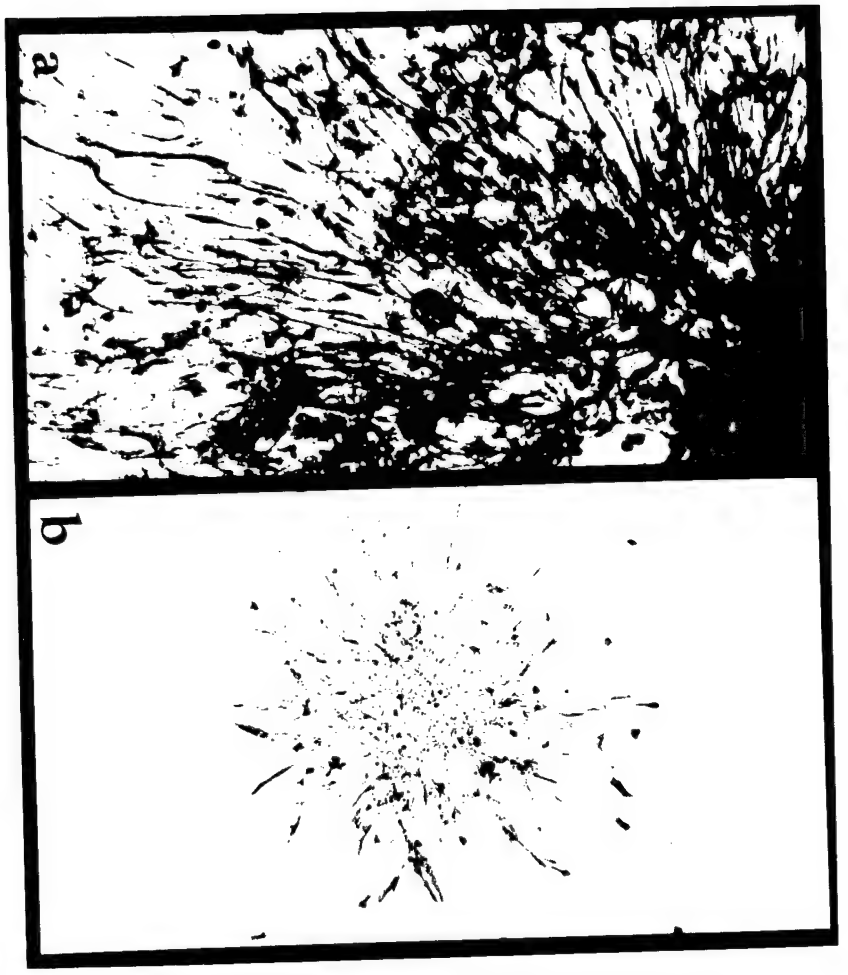
Figure 5. Representative examples of various phenotypic markers in cryopreserved MPC-C9 cells. The cells were thawed and plated at $1X10^6$ cells/cm^2. The cultures were stained for various DA markers including TH (A), the DA transporter (B), DA itself (C), and DDC (D). As was true in the lower magnification photomicrographs of the cultures (see Figs. 1 and 2), most of the cells in the cultures were found in dense clusters that did not reveal cellular morphology very well. Isolated small clusters outside the large clusters were thus photographed here. In the clone studied for DA neuron markers, all the clusters outside the neurospheres were immunoreactive. Note the varicosities and irregular shaped soma characteristic of DA neurons in culture (arrows in C)). In addition, cells that were GFAPir (E) and nestin-ir (F) were occasionally found. Note that the nestin-ir cell depicted in F exhibits primarily a surface reactivity with an un-reacted soma core typical of cells transitioning from a nestin-ir cell to a committed phenotype (Magnification bar = 12 μm).

Figure 6. Cytokine-treated mesencephalic progenitors survive transplantation and ameliorate amphetamine-induced rotational behavior in the rat model of Parkinson's disease. (A) Implants of cytokine-treated progenitors (Cvt.Prog.; N=7) reduced rotational behavior to the same extent as freshly harvested fetal ventral mesencephalic (Fetal VM) cells (N=7), but at a slower rate of recovery. Fetal VM grafts exhibited significant reductions in rotation as compared to their own baseline beginning at 2 weeks after grafting that continued through the 8 weeks of study. Cytokine-treated progenitor grafts showed reduced rates of rotation compared to their baseline at 6 and 8 weeks post-grafting. At weeks 4-8 for fetal VM, and weeks 6 and 8 for cytokine-treated progenitors these groups exhibited significantly reduced rates of rotation as compared to both sham operated controls (n=5) and rats receiving grafts of progenitor cells that were not exposed to cytokines (control progenitors; N=6). These latter two control groups showed no amelioration of their rotational asymmetry. (* significant difference from baseline. + significant difference from two control groups.) (B) Counts of TH-positive neurons in grafts at an interval of 210μm yielded a an uncorrected cell count mean and s.e.m. of 660.4 +/- 82.8 cells for fetal VM grafts (N=7), 173.7 +/- 35.4 cells for cytokine-treated progenitor cell grafts (N=7), and 0 cells for progenitor grafts not exposed to cytokines (N=6). Cell counts for the 2 grafts in each animal were combined for statistical analysis. (* significant difference from Cvt. Prog. and NCvt. Prog. + significant difference from NCvt. Prog.) (C-E) Representative TH-stained tissue sections through transplants of fetal VM (C), cytokine-treated progenitors (D) and progenitors not exposed to cytokines (E). Readily apparent are the decreased yield of TH-positive neurons in cytokine-treated progenitor grafts as compared to fetal VM grafts, and the absence of these neurons in progenitor grafts not exposed to cytokines (staining in panel E is non-specific and associated with residual elements of blood). In addition, a range of TH-positive fiber staining provided by the grafts is visible, with best reinnervation provided by fetal VM grafts, intermediate reinnervation provided by cytokine-treated progenitors, and an absence of fiber staining in the denervated striatum of rats implanted with progenitors not exposed to cytokines. (Bar in panel E = 100μm.)

Table 1. Percentage of THir cells in various clones from Mesencephalic progenitor cells. After three passages, 3-7 neurospheres from each clone were taken from the clonal expansion procedure, triturated, and plated onto two poly-l-lysine coated 48 well plates and exposed to complete media containing IL-1α (100 pg/ml). After six days, the total number of cells in each well of the two replicates/clone was counted and compared with the total number of THir cells in that well to determine the percentage conversion rate[12] .

Clone	% THir Cells	Clone	% THir Cells	Clone	% THir Cells	Clone	% THir Cells
MPC-C1	6	MPC-C7	5	MPC-C13	8	MPC-C19	2
MPC-C2	26	MPC-C8	98	MPC-C14	21	MPC-C20	23
MPC-C3	78	MPC-C9	98	MPC-C15	23	MPC-C21	3
MPC-C4	3	MPC-C10	5	MPC-C16	2	MPC-C22	74
MPC-C5	77	MPC-C11	72	MPC-C17	26	MPC-C23	47
MPC-C6	76	MPC-C12	44	MPC-C18	21	MPC-C24	43

FIGURE 1



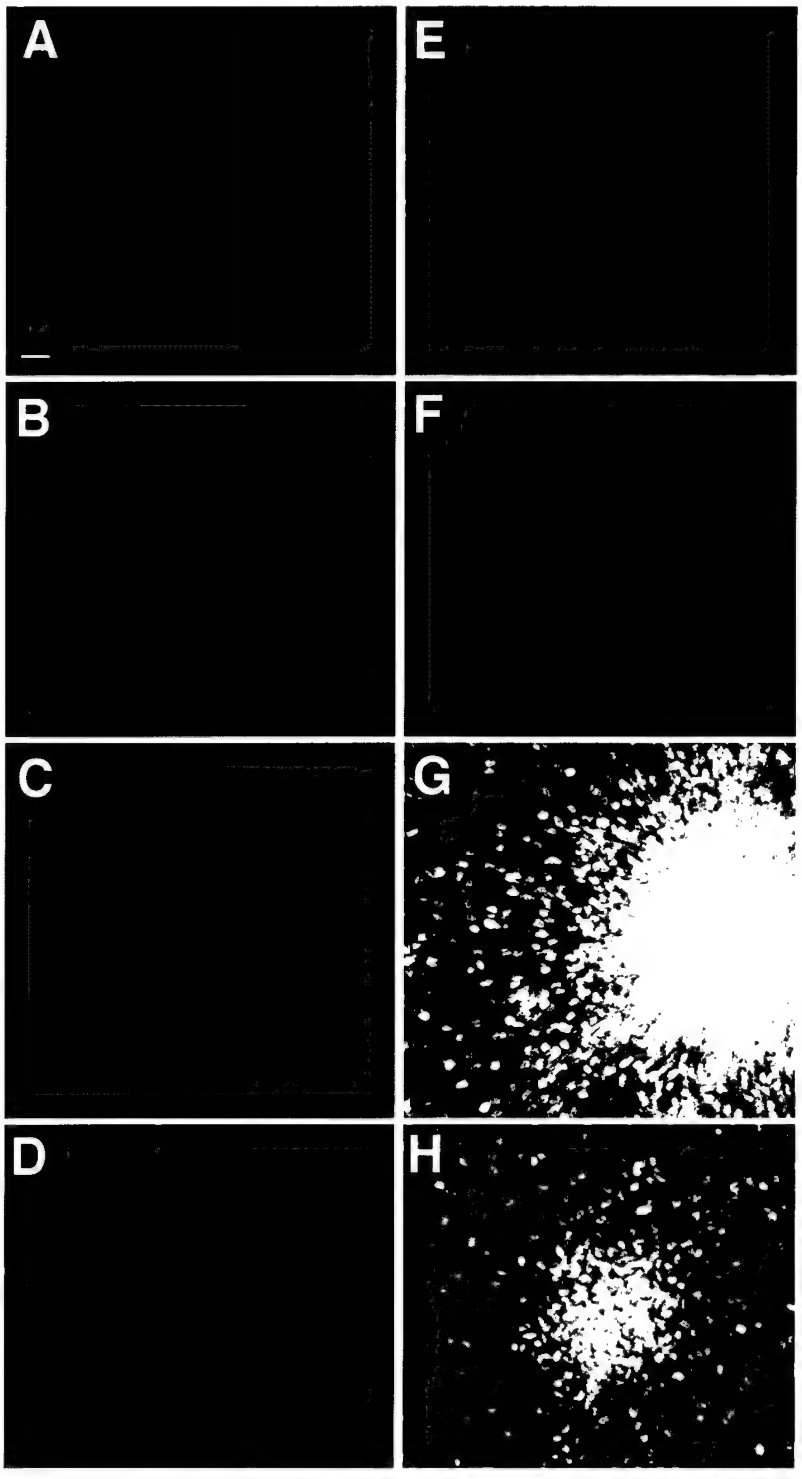


FIGURE 2

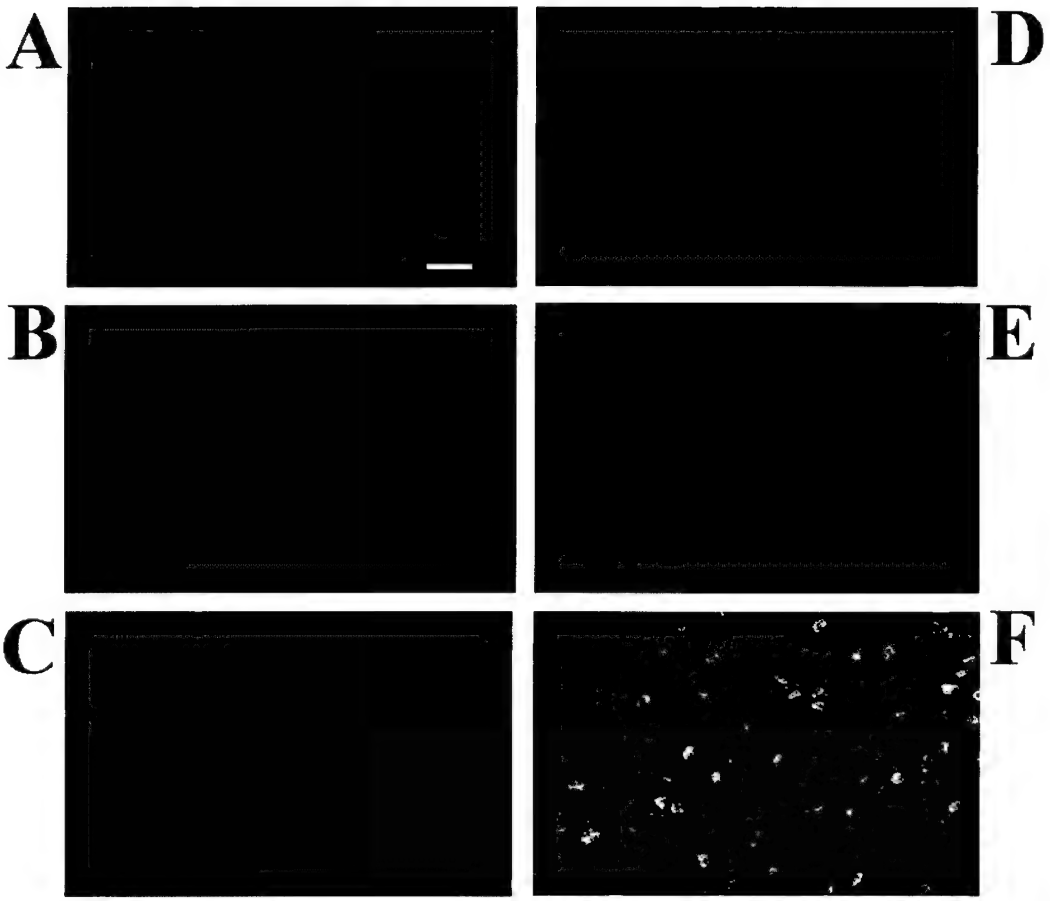


FIGURE 3

FIGURE 4

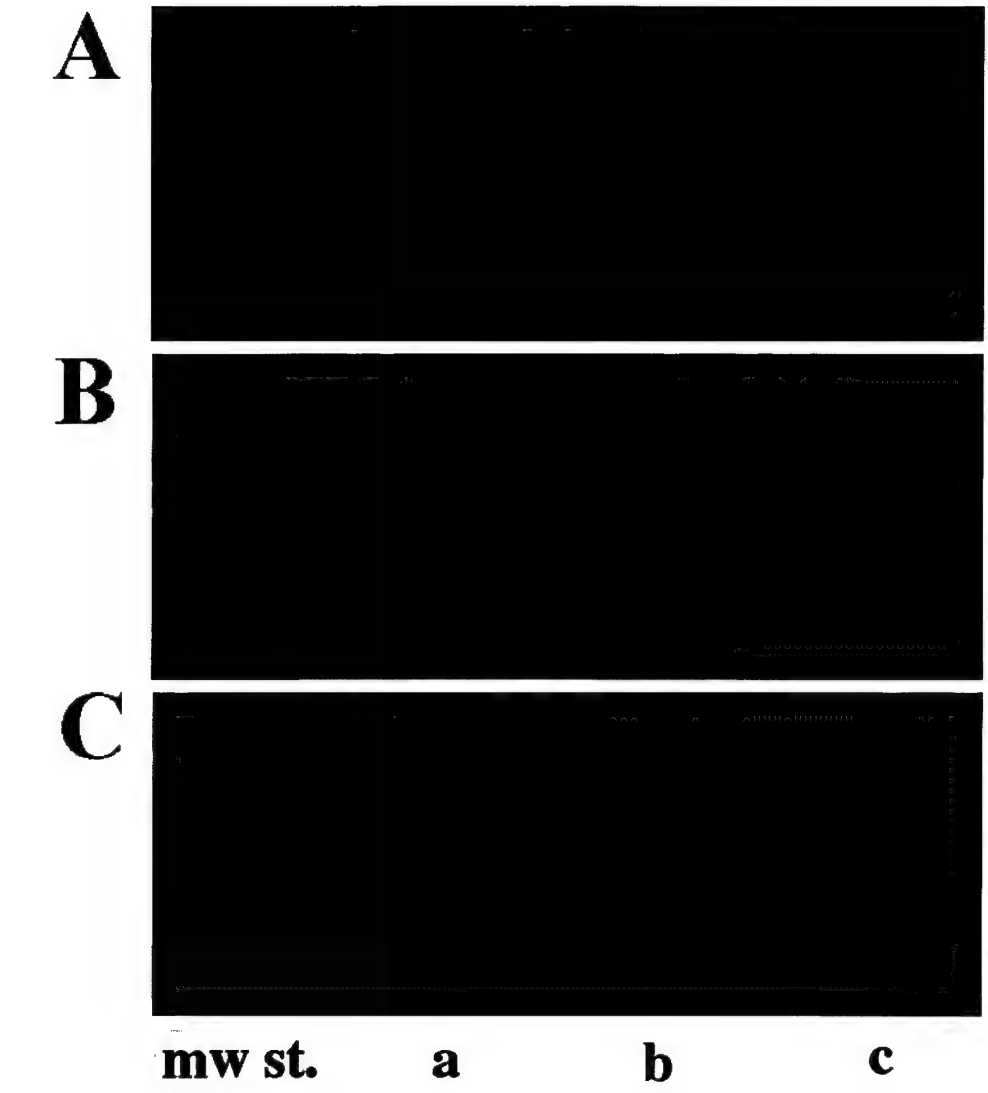
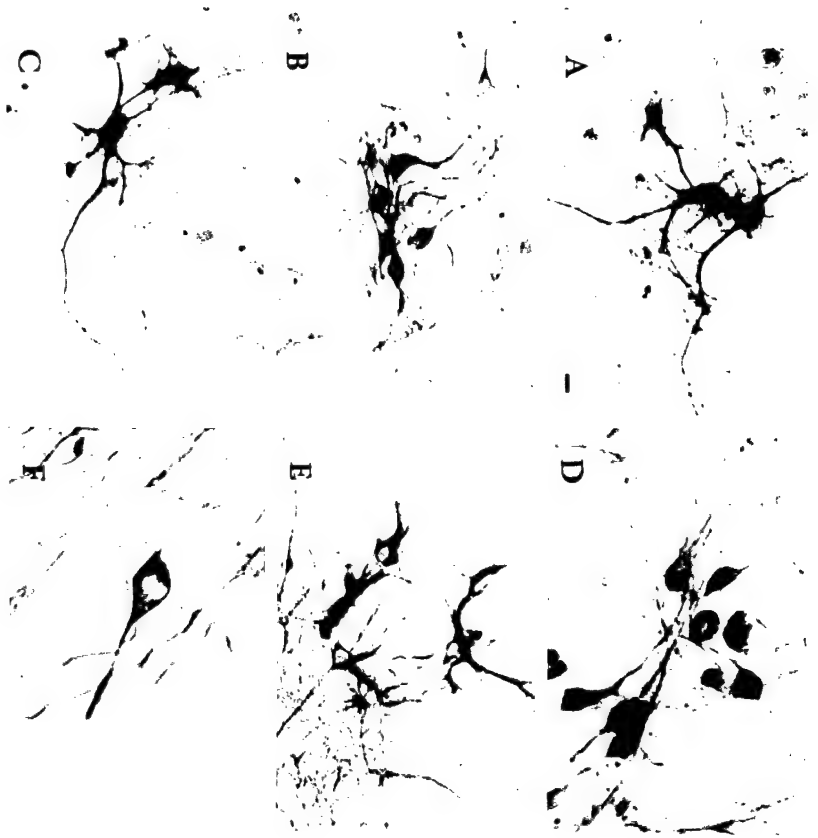


FIGURE 5



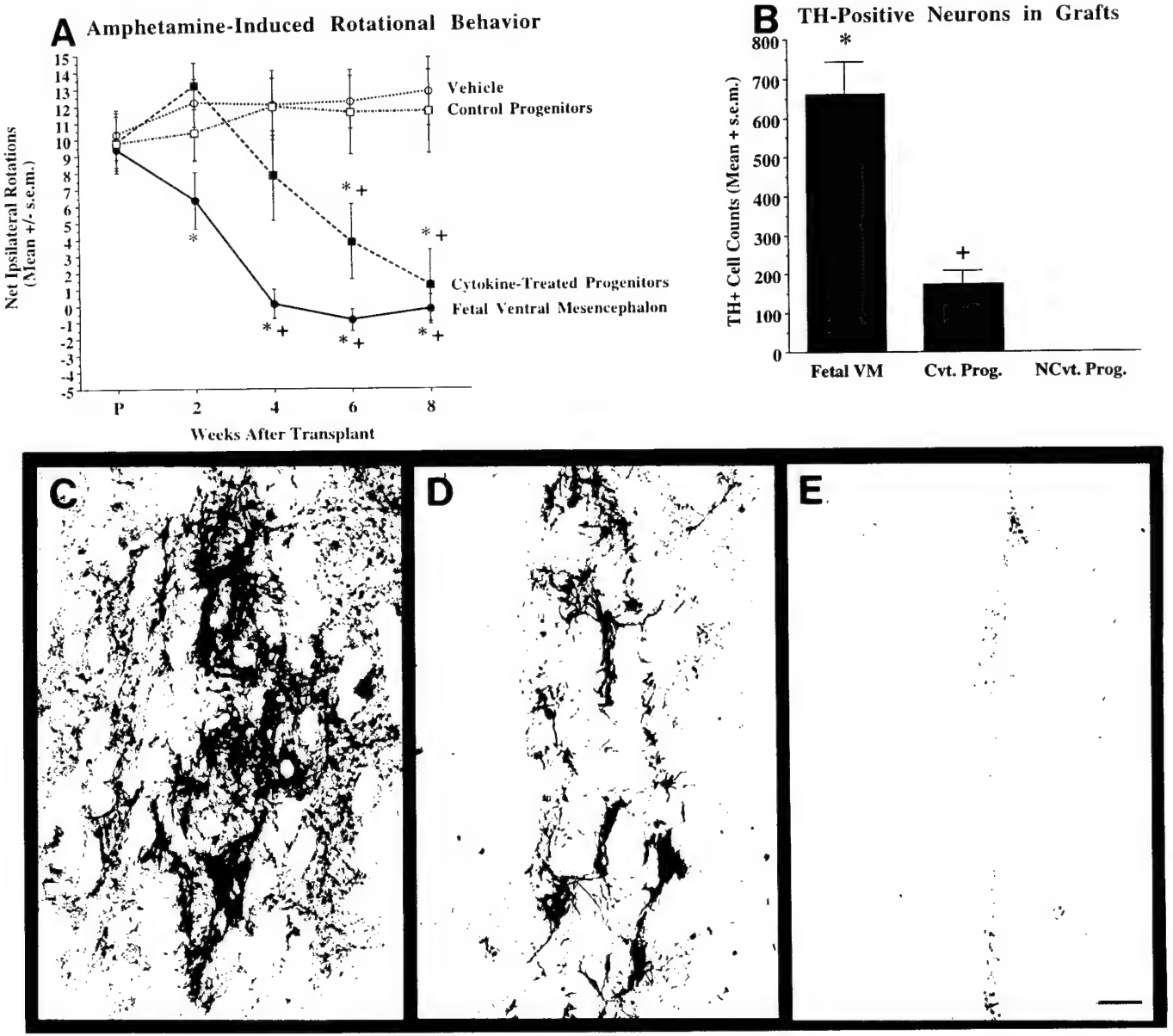

A
Amphetamine-Induced Rotational Behavior
Net Ipsilateral Rotations
(Mean +/- s.e.m.)
Vehicle
Control Progenitors
Cytokine-Treated Progenitors
Fetal Ventral Mesencephalon
P
2
4
6
8
Weeks After Transplant
B
TH-Positive Neurons in Grafts
TH+ Cell Counts (Mean + s.e.m.)
Fetal VM
Cvt. Prog.
NCvt. Prog.
C
D
E

FIGURE 6

133.1

INTERLEUKIN-1 INDUCES Nurr-1 EXPRESSION AND THE DA PHENOTYPE IN MESENCEPHALIC PROGENITOR CELLS. P.M. Carvey* and Z.D. Ling. Depts. of Pharmacology and Neurological Sciences, Rush-Presbyterian St. Luke's Medical Center, Chicago IL 60612.

Progenitor cells from the subependymal region of the mesencephalon of E14.5 rats can be converted to DA neurons by the cytokines Interleukin 1 (IL-1), leukemia inhibitory factor (LIF), IL-11, and glial cell line-derived neurotrophic factor (GDNF). Although LIF and GDNF were necessary for the maturation of the DA neurons, they were ineffective at progenitor cell conversion to the DA phenotype without IL-1. We subsequently demonstrated that IL-1 turned on the expression of gp130 in MAP-2 immunoreactive cells suggesting that IL-1 initiated a transcriptional event leading to gp130 mediated responsiveness. Since Nurr-1, an orphan receptor superfamily of nuclear receptors, has been shown to be essential for the development and maintenance of the DA neuron phenotype, we sought to determine if IL-1 similarly turned on the expression of this factor. In this particular study, clonally expanded progenitor cells were exposed to IL-1 (200 pM) or control media for up to 6 days, fixed, stained, and assessed using confocal microscopy. Nurr-1 was present in control cultures but at low levels. In contrast, IL-1 dramatically increased Nurr-1 expression which was confirmed by the Western blot. Nurr-1 was co-localized with tyrosine hydroxylase immunoreactive (THir) cells although many of the Nurr-1ir cells were not THir. However, all MAP-2ir cells were Nurr-1ir. None of the Nurr-1ir cells were GFAPir suggesting that Nurr-1 was expressed only in neurons: These data suggest that IL-1 increases the expression of Nurr-1 and that this expression is correlated with the development of the DA neuron phenotype. (Support by DOD grant DAMD17-98-1-8629)

133.2

ESTRADIOL REDUCES LEVODOPA- AND BACTERIAL LIPOPOLYSACCHARIDE-INDUCED DA NEURON LOSS. Z.D. Ling[1,2]* J.W. Lipton[1,2,3], C.W. Tong[1], and P.M. Carvey[1,2]. Depts. of Pharmacology[1], Neurological Sciences[2], and Pediatrics[3], Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL 60612.

The incidence of Parkinson's disease (PD) is higher in men than in women and estrogens are known to regulate DA neuron function. We examined the effects of estradiol on DA neurons in normal primary mesencephalic cultures as well as cultures exposed to levodopa which is known to kill DA neurons through oxidant stress. We also evaluated estradiol in cultures exposed to bacterial lipopolysaccharide (LPS), an endotoxin that stimulates glial cells to produce tumor necrosis factor alpha (TNF-α) which induces DA neuron loss through a receptor mediated mechanism. The number of tyrosine hydroxylase immunoreactive (THir) cells served as an index of DA neuron survival. Estradiol alone significantly increased DA neuron survival ($F=9.645$; $P=0.014$), promoted sprouting of DA neurons, and dose-dependently attenuated levodopa (50 μM) induced DA cell loss ($F=32.263$; $P=0.001$). Pre-treatment of mesencephalic cultures with 1 μM estradiol did not alter LPS-induced TNF-α release in culture, but suppressed LPS-induced DA neuron loss ($F=14.746$; $P<0.001$). These results suggest that estrogen acts as a neuroprotectant to DA neurons potentially playing a role in maintaining the normal morphology of the DA neuron while increasing resistance to oxidant stress and receptor-mediated toxicity. Estrogen replacement therapy in post-menopausal women may therefore reduce or delay the development of Parkinson's disease in women. (Supported by the Parkinson's Disease Foundation).

133.3

THE EFFECTS OF ACTIVATED MICROGLIA ON NIGROSTRIATAL DOPAMINERGIC SYSTEM IN RAT. BK Jin[1]*, IK Ryu[1], I Kim[1], MH Kim[1], KK Cho[2], YW Ahn[2], HK Pyo[3] EH Joe[3], SU Kim[1,1]Brain Disease Research Center, [2]Department of Neurosurgery, [3]Department of Pharmacology, Ajou University School of Medicine, Suwon 442-749, Korea.

Activated microglia has been known to play a pathogenic role in the neurodegenerative diseases including Parkinson's disease(PD). In the present study, neurotoxic effects of activated microglia were investigated in rat nigrostriatal dopaminergic system. Cultured rat microglia activated by lipopolysaccharides(LPS) or LPS alone was injected into substantia nigra(SN) in Sprague-Dawley rats. The degree of microglia activation was measured by amount of nitric oxide(NO) released into culture media. Microglia exposed to LPS for 48 hr produced the highest amount of NO although transplantation of these activated microglia resulted in only mild and transient rotational behavior following amphetamine administration. In contrast, in animals receiving nigral injection of LPS, rotational behavior was much more intense and dose-dependent (0.5, 1, 5, 10 mg/mℓ). Immunocytochemical examination of brain in these experimental animals demonstrated that there were a profound loss of dopaminergic neurons in SN and terminals in striatum evidenced by tyrosine hydroxylase(TH) staining. Reduction in the number of TH+ neurons in SN was dose-dependent and increases in the number of activated microglia and astrocyte were also demonstrated. These results support the hypothesis that activated microglia could contribute to the degeneration of nigrostriatal dopaminergic system in PD.

133.4

CEP-1347/KT-7515, A SAPK/JNK PATHWAY INHIBITOR REVERSES MPP^+ INDUCED TOXICITY IN MULTIPLE IN VITRO NEURONAL SYSTEMS. J. R. Mathiasen*, B. A. W. McKenna, L. Lu, R. W. Scott and D. Bozyczko-Coyne. Dept. of Neurobiology, Cephalon, Inc.; West Chester, PA 19380.

Parkinson's Disease (PD) is characterized by a loss of nigro-striatal dopamine neurons and PD patients have widespread decreases in mitochondrial function. Loss of dopamine neurons in PD has been associated with apoptotic cell death. There is also evidence that activation of the stress-activated protein kinase (SAPK)/c-jun N-terminal kinase (JNK) pathway leads to apoptotic cell death. *In vivo* models of PD utilize the selective nigro-striatal dopaminergic neurotoxin, MPTP. MPTP is oxidized in glial cells to the toxic product MPP^+ that is selectively taken up into dopamine neurons and induces death that has been linked to apoptosis. Previous studies have shown protection in an MPTP mouse model of PD with CEP-1347/KT-7515, (bis-ethylthiomethyl analog of K-252a) [Saporito, et. al.; J Pharmacol Exp Ther 1999 Feb; 288(2):421-7], an inhibitor of the SAPK/JNK pathway [Maroney, et. al. J. Neuroscience 1998 18:104-111]. Here, we used neuronal cells that are responsive to the neurotoxic effects of MPP^+ in a functional assay of benztropine-sensitive [^{3}H]-dopamine uptake and a survival assay measuring lactate dehydrogenase (LDH) release: 1. In rat E14 ventral mesencephalic (VM) primary neuronal cultures, MPP^+ (10 uM) induced ~ 50% decrease in [^{3}H]-dopamine uptake. CEP-1347/KT-7515 protected up to 30% of the MPP^+ induced decrease in [^{3}H]-dopamine uptake and increased tyrosine hydroxylase (TH) immunopositive neurons. 2. In differentiated human SH-SY5Y cells (dopaminergic, neuroblastoma) 3 mM MPP^+ induced cell death ~ 5-fold over basal as measured by LDH release. CEP-1347/KT-7515 prevented this MPP^+ induced cell death in a concentration-dependent manner with maximal efficacy (~50 % rescue) achieved at 30 nM. CEP-1347/KT-7515 also maintained normal SH-SY5Y cell morphology versus MPP^+ induced changes. The SAPK/JNK inhibitor, CEP-1347/KT-7515, protection against MPP^+ induced toxicity implicates this signaling pathway in the pathophysiology of PD. Together, data support therapeutic potential for CEP-1347/KT-7515 in the treatment of Parkinson's Disease.

133.5

HALOPERIDOL INDUCES PARKINSON'S DISEASE-LIKE CELL LOSS IN SUBSTANTIA NIGRA IN AGED RATS. A.Kolbasnik, S. Garside, A. Levinson, P.I. Rosebush*M.F. Mazurek. Departments of Neurology, Psychiatry and Behavioural Neuroscience, McMaster University Medical Centre, Hamilton, ON. L8N 3Z5

Loss of midbrain dopamine neurons is the neuropathological hallmark of Parkinson's Disease (PD). We recently reported that the antipsychotic drug haloperidol (HAL) induces reversible downregulation of dopamine neurons in substantia nigra (SN) in young rats. (Neuroscience 84:201-211, 1998). We studied whether HAL might cause PD-like cell death in older animals. Male Sprague-Dawley rats received either oral HAL 1.8 mg/kg/day or normal drinking water for 3 weeks. Subsequent examination of the SN showed that younger HAL-treated animals (6 months of age) had a 35% reduction of tyrosine-hydroxylase (TH)-immunoreactive cell counts 3 weeks after discontinuation of the drug ($p<.001$), with recovery almost to control levels by 7 weeks withdrawal. By contrast, older HAL-treated animals (18 months of age), had a persistent 39% reduction of TH-positive cell counts 7 weeks after stopping the drug ($p<.001$) and a 30% loss of Nissl-stained cells in medial SN ($p<.001$). Further analysis showed TUNEL-positive cells ($\bar{x}=8.2\pm0.9$/section) in the SN of older HAL-treated rats, but none in the older control rats. These results show that: (1) The neuroleptic drug HAL can induce reversible suppression of dopaminergic cells in the SN of younger animals. (2) A 3-week course of HAL can cause apoptotic cell death in nigral cells in older rats. Implication: Antipsychotic drugs may be capable of causing Parkinson's disease in older individuals.

Supported by NSERC and Schizophrenia Society of Ontario.

133.6

NICOTINE PARTIALLY PROTECTS DOPAMINERGIC CELLS FROM MPP^+ NEUROTOXICITY *IN VITRO*. G. Jeyarasasingam* and M. Quik. The Parkinson's Institute, Sunnyvale, CA 94089.

Epidemiological studies show an inverse correlation between cigarette smoking and the incidence of Parkinson's disease. Accumulating evidence suggests that nicotine, a major component of cigarette smoke, may be responsible for such neuroprotection *in vivo*. To test whether this may be due to an effect of nicotine at the level of the substantia nigra, we have examined the potential neuroprotective effects of nicotine against the dopaminergic neurotoxin MPP^+ on ventral mesencephalic neurons *in vitro*. Receptor binding studies were first done to determine whether nicotinic receptors are present on mesencephalic neurons in culture. Robust binding of both ^{3}H-epibatidine, which interacts with α2-α5 containing receptors and ^{125}I-α-bungarotoxin, which interacts with α7 receptors, was observed in culture. We then tested the ability of nicotine to attenuate MPP^+ induced toxicity by assessing the number of cells immunoreactive for tyrosine hydroxylase (TH positive). Incubation with 3 μM MPP^+ for 48 hours resulted in a 70% reduction in the number of TH positive neurons as compared to control cultures. However, cultures treated with 10^{-5} M nicotine for 24 hours prior to MPP^+ exposure exhibited only a 53% decline in the number of TH positive cells as compared to control. These results show that nicotine exposure significantly prevents MPP^+ induced degeneration of dopaminergic nigral neurons. (Supported by TRDRP 7FT-0010).

7-2

GENERATION AND TRANSPLANTATION OF HUMAN NEURAL STEM CELL-DERIVED CATECHOLAMINERGIC NEURONS INTO A PARKINSONIAN ANIMAL MODEL.

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A continuous expandable source of human dopaminergic neurons would be ideal for reconstructive cell transplantation therapy in Parkinson's disease (PD). In an effort to develop such a cell line, we have first demonstrated that a glial-derived conditioned medium (CM) co-operates with FGF2 to induce tyrosine hydroxylase (TH) expression *in vitro* in the embryonic and adult murine forebrain ventricular zone-derived precursors (Daadi and Weiss, 1999). An expandable neural stem cell line was then established from the embryonic human CNS through epigenetic stimulation with mitogenic growth factors (Vescovi et al., 1999). This stem cell line maintained its multipotentiality throughout numerous passages and gave rise to neurons, astrocytes and oligodendrocytes. When these human stem cell progeny were cultured under differentiating conditions and treated with FGF2 and CM, they expressed TH within 24 hours after plating. The number of TH-immunoreactive (TH-IR) cells gradually increased and reached 8% of the total cell population at day 7 and remained stable thereafter. After 7 days in culture, nearly all the TH-IR cells co-expressed the neuronal marker TuJ1, the dopamine transporter (DAT) and D2 receptor subtype. We then asked the question whether or not these TH induced neurons could maintain this newly acquired phenotype after transplantation into the 6-OHDA rat animal model of PD. Our results show that after a three-month post-transplantation survival period, induced neurons did not express TH within the lesioned striatum. Possible explanations and speculations will be discussed.

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PROGENITOR CELLS CONVERTED TO DA NEURONS BY CYTOKINES CAN BE GRAFTED AND CRYOPRESERVED.

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We previously reported that mesencephalic progenitor cells (MPCs) would convert into cells immunoreactive for several phenotypic markers of the dopamine (DA) neuron following incubation in cytokine supplemented media which included interleukin-1 (IL-1), IL-11, leukemia inhibitory factor (LIF), and glial cell line-derived neurotrophic factor (GDNF). In order to determine if these cells were functional DA neurons and could be used as a practical source of cells for neural grafting, we assessed their ability to attenuate rotation in rats with unilateral 6-hydroxydopamine lesions and their stability following cryopreservation. MPCs were clonally expanded in epidermal growth factor (EGF) using the limiting dilutions technique. The clone expressing the highest percentage conversion rate (98%) to tyrosine hydroxylase immunoreactive (THir)cells in response to IL-1 was exposed (5h) to the cytokine mixture, washed, and grafted into the ipsilateral striatum of lesioned animals and subsequently assessed for amphetamine-induced rotation (5 mg/kg). These animals were compared with animals grafted with cloned cells not exposed to cytokines, animals receiving normal fetal grafts, and sham controls. Both the cytokine-exposed clones and fetal grafted animals exhibited significant, progressive rotational attenuation (over 8 weeks) relative to both control groups ($F=7.46$; $p < 0.005$) and had robust THir cell survival in the graft site. Following cryopreservation (10% DMSO) the clone remained immunoreactive for 4 different phenotypic markers of DA neurons. These data suggest that MPCs can be cryopreserved and serve as an unlimited, on demand source of cells for grafting in Parkinson's disease. The results further suggest that hematopoietic cytokines play a central role in the normal development of DA neurons. (DAMD17-98-1-8629 and AG10851)

FIGURE 1

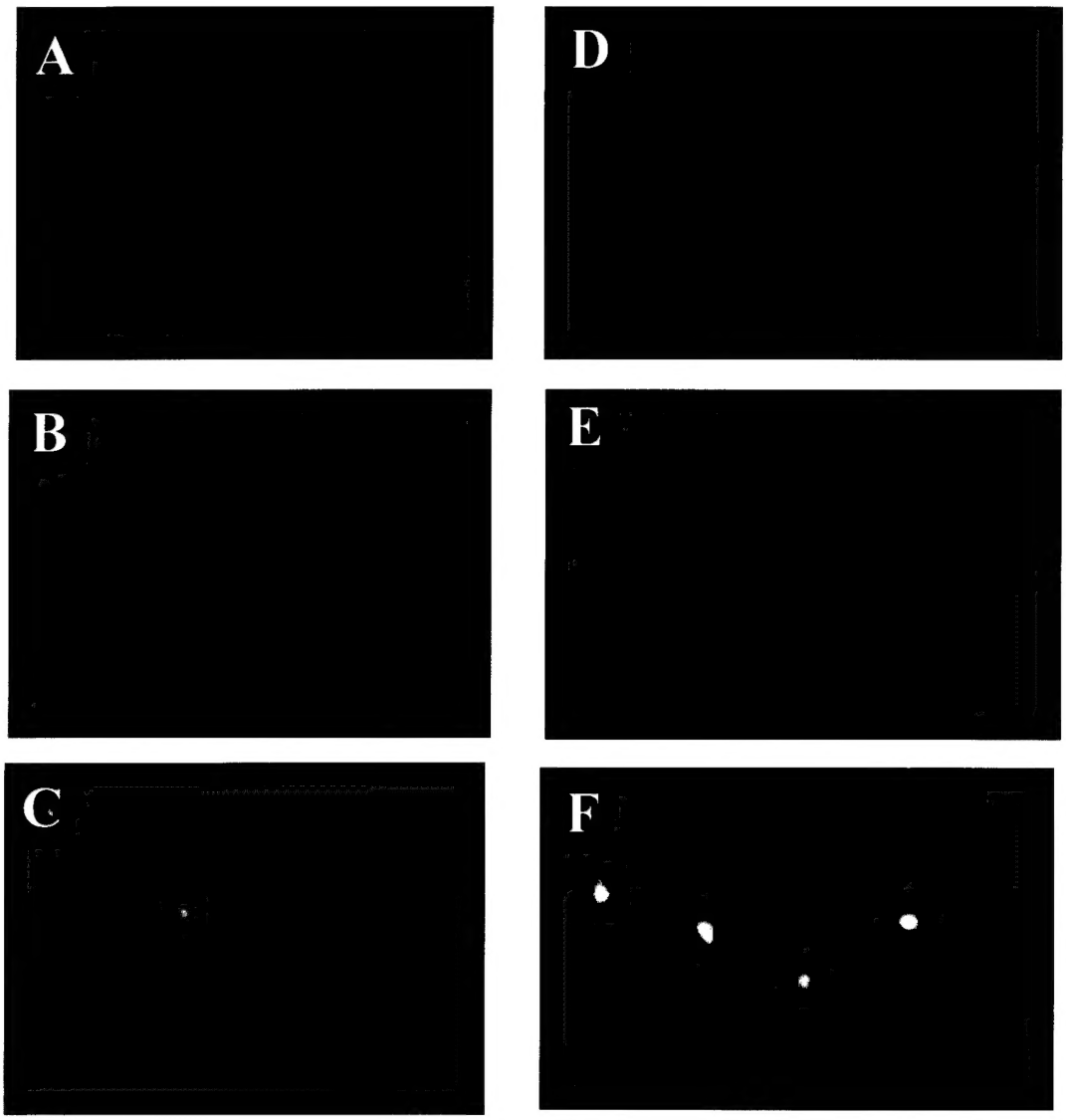
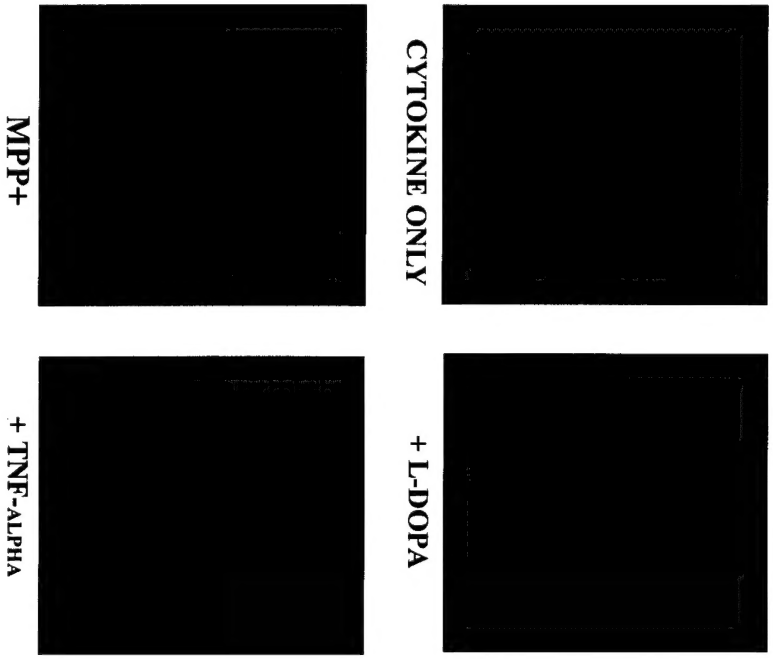


FIGURE 2



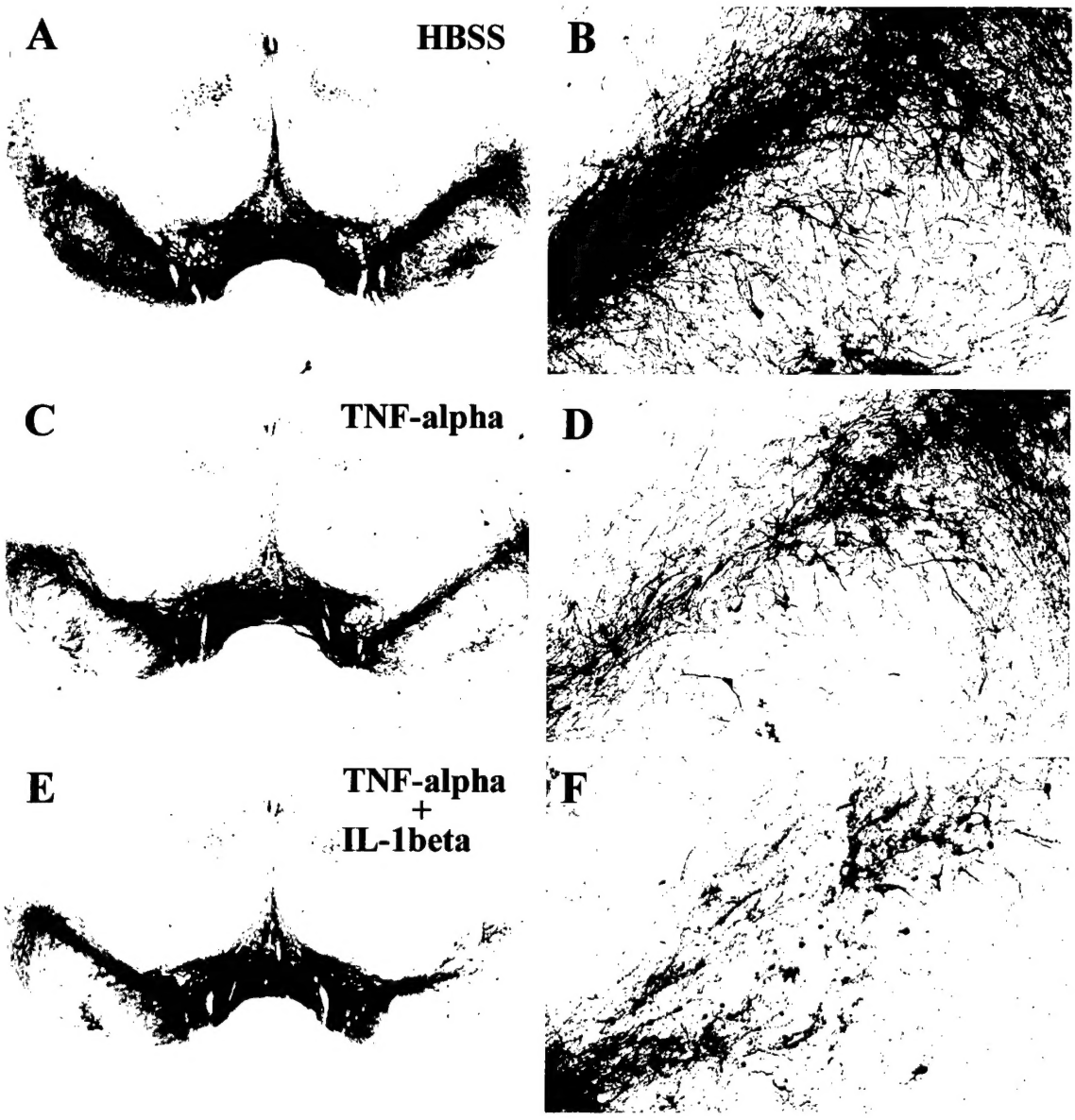


FIGURE 3

FIGURE 4